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(54) Title: BONE TARGETING OF BIODEGRADABLE DRUG-CONTAINING NANOPARTICLES

(57) Abstract: This invention provides use of a composition for the manufacture of a medicament for modifying a cellular response in a mammal. The composition to be used an effective amount of biodegradable nanoparticles for administration to a mammal, each of said nanoparticles comprising an active agent, a biodegradable polymer, and a bone targeting agent administering to a mammal an effective amount of a composition comprising a compound absorbed in a biodegradable nanoparticle which is attached to a bone targeting agent. The invention also provides the use of said composition for the manufacture of a medicament for modifying a cellular response in a mammalian cell comprising contacting the mammalian cell with biodegradable nanoparticles. The invention provides the use of said composition for the manufacture of a medicament for delivering an exogenous substance in addition a composition is disclosed comprising the exogenous substance absorbed into a biodegradable nanoparticle is covalently attached to a bone targeting agent, and methods administration to a mammal. The invention also provides a composition biologically active or therapeutic agent of compound a bone

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BONE TARGETING OF BIODEGRADABLE DRUG-CONTAINING NANOPARTICLES

FIELD OF THE INVENTION

[0001] This invention pertains to compositions and methods for the targeted and controlled delivery of active agents to mammalian cells, for example, bone and bone marrow cells employing nanoparticles.

BACKGROUND OF THE INVENTION

[0002] Targeted delivery of active agents, e.g., therapeutic substances, to a specific location of the body is a goal that has met with only limited success. One of the goals in the treatment of disease is to specifically deliver the therapeutic agent exclusively to the area requiring treatment. Extensive effort has been put forth in rational drug design to produce a compound that will selectively treat the specific ailment, but the biological variety that exists within a living organism means the drug usually does not interact exclusively with the tissue requiring treatment. The classical example of this challenge is the treatment of cancer with chemotherapeutic agents. Chemotherapy usually focuses on killing the active cancer cells, typified by uncontrolled growth, at a faster rate than it kills the healthy cells that may coincidently be growing at the same time.

[0003] Another goal in the treatment of disease is the controlled release of the therapeutic substance over an extended period of time, in order to provide a sustained treatment using a single dose rather than multiple doses. Several products on the market are available that use biodegradable products to release a drug into the body over a specific period of time. For example, parenteral depot systems, such as Lupron Depot, Nutropin Depot, and Trelstar Depot listed in the FDA Orange Book, are products that provide a controlled release from poly(lactic -*co*-glycolic) acid (PLGA) microparticles of a therapeutic agent over a period of one month or more. However, the injection of this formulation is localized, typically a parenteral injection, and the drug is released into the blood stream and distributed throughout the body rather than exclusively to the area of requiring treatment.

[0004] Substantial work has been done on microparticles, and the commercial Depot products are typically of a size on the order of microns. Smaller nanoparticles have only recently been described and result from the realization that sub-micron particles could find utility in particular drug applications (Jain, *Biomaterials*, *21*, 2475 (2000)).

[0005] The combination of targeted delivery and controlled release of a therapeutic agent at a specific location of the body has only recently been explored in a viable manner by the use of biodegradable nanoparticles targeted to a specific tissues in the body. For

on the order of 10 nanometers, and did not provide for the controlled release of a therapeutic agent. The iron-containing nanoparticles were exposed to bisphosphonates in aqueous solution, and physiochemical surface adsorption was postulated. Some limited localization was found in male Wistar rats. Using radiolabeled iron-59, the animal studies (18 hours after injection) indicated at best 6% of injected dose in the skeletal system with almost half going to the spleen and liver. The attachment of the bisphosphonate to the nanoparticle was not optimized or characterized. Roberts and Kozlowski (U.S. Patent 6,436,386) describe a PEG molecule covalently attached to both a bisphosphonate and a drug candidate for targeting bone tissue. The product was not described to form nanoparticles nor was the compound described as a component for forming nanoparticles.

[0008] The foregoing shows that there exists a need for particles, especially nanoparticles, for the targeted delivery and controlled release of therapeutic agents to the bone and bone marrow of an animal or human. The present invention provide such particles. The advantages of the invention, as well as additional inventive features, will be apparent from the description of the invention provided herein.

BRIEF SUMMARY OF THE INVENTION

[0009] The invention provides a method of modifying a cellular response in a mammal comprising administering to the mammal an effective amount of biodegradable nanoparticles, each of said nanoparticles comprising an active agent, a biodegradable polymer, and a bone targeting agent. The invention also provides a method for modifying a cellular response in a mammalian cell comprising contacting the mammalian cell with biodegradable nanoparticles.

[0010] In addition, the invention provides a method of delivering an exogenous substance to a mammal The method comprises administering to the mammal an effective amount of biodegradable nanoparticles comprising the exogenous substance, a biodegradable polymer, and a bone targeting agent.

[0011] The invention provides a composition comprising a active agent, a biodegradable nanoparticle, and a bone targeting agent. The invention further provides for a process for preparing a biodegradable nanoparticle comprising a active agent, a biodegradable polymer, and a bone targeting agent.

BRIEF DESCRIPTION OF THE DRAWINGS

[0012] Figure 1 depicts the chemical structure of EDTMP.

[0013] Figure 2 depicts the chemical structure of DOTMP.

[0014] Figure 3 depicts the chemical structure of ABDTMP.

[0015] Figure 4 depicts the chemical structure of BAD.

[0033] Figure 22A and 22B are graphs illustrating the release profiles of nanoparticles containing doxirubicin and epirubicin, respectively.

[0034] Figure 23 depicts reaction scheme for preparing a PEG-modified polyester, AJ, or a phosphonic acid modified polyester, AI.

[0035] Figure 24 depicts a scheme for preparing an aminodiphosphonic acid-PEG-PLGA, AM, for the use in nanoparticle preparation.

[0036] Figure 25 depicts a reaction scheme for preparing an aminotetraphosphonic acid-PEG-PLGA, AP, for the use in nanoparticle preparation.

DETAILED DESCRIPTION OF THE INVENTION

[0037] The invention provides biodegradable nanoparticles that are sufficiently modified with anionic calcium binding moieties in order to target or deliver the nanoparticles to a selected tissue, cell, or organ, e.g., the bone, of an animal. Contained within the nanoparticles are one or more active agent, e.g., therapeutic agents, that can modify a cellular response in the bone or bone marrow of a patient. Such delivery of active agents to the bone can be used as a sensitizer to enhance the effects of chemotherapy or radiation treatment of bone and marrow diseases, or to deliver chemotherapeutic agents to the bone and bone marrow. Alternatively, the therapeutic agent can be a chemoprotectant to prevent bone marrow suppression during chemotherapy or radiation treatment of nonbone/nonmarrow diseases, or can deliver agents that encourage bone growth or regrowth. By targeting the biodegradable nanoparticles to the bone, the therapeutic agent's effect can be localized and the delivery of the drug at this site controlled over a specific period of time.

[0038] One aspect of the invention is the controlled delivery of a drug at one or more selected rates over an extended period of time. The delivery can be via oral, transdermal, or parenteral (injectable or implantable) routes. These controlled release systems release enough drug to maintain the drug level in the body at an effective therapeutic concentration over a long period of time. The advantages of such release systems in general are the avoidance of toxic or ineffective drug levels, the most efficient use of the drug itself, and fewer drug doses than with systems of conventional administration. The drug can be released at a constant period over time, at a pusatile rate of delivery, or at different rates, e.g., an initial delivery of a bolus of drug followed by a slower controlled rate of delivery.

[0039] In an embodiment of the invention, a therapeutic agent is delivered by a biodegradable nanoparticle that contains the therapeutic agent. By "biodegradable" is meant a compound that can be decomposed, degraded, or otherwise destroyed by biological or biochemical processes. The products of these biodegradable polymers may be completely broken down and removed from the body by normal metabolic pathways.

[0042] In another embodiment, the biodegradable nanoparticle comprises poly(ethylene glycol) or poly(ethylene oxide), commonly known as PEG or PEO, which is a polyether formed either from ethylene glycol or ethylene oxide as a monomer. The molecular weight of the PEO or PEG can be any suitable weight, e.g., it can range from as low as 400 to as high as 5,000,000. Preferably, the molecular weight is from about 700 to about 100,000, more preferably from about 1000 to about 20,000, and even more preferably from about 3000 to about 5000. PEG is currently being used in drug delivery for suppositories, prostaglandin formulations, and contraceptive sponges, and as a wound healing laminate. Although PEG is not degraded in the body, it has been shown to be safe for biological applications, with no detectable toxic or cumulative effects of intravenous injection of PEG even after repeated doses ranging up to 90 mg/kg per day. Many PEGs can be prepared with a functional group that provides for attachment of another moiety. These activated PEGs have been designed for attachment to lysine amino groups, making them ideal for use with proteins, peptides, and enzymes. Protein-PEG conjugates are more stable to proteolyses and denaturation than the native proteins. Modified PEGs provide increased thermal stability and aqueous solubility, e.g., when modified with immunoglobulin G.

[0043] In the invention, PEG can be incorporated into the nanoparticle by any suitable approach, e.g., as a block copolymer of the biodegradable polymer graft or as an attachment (e.g., covalent) to the nanoparticle or its surface, as a blend of PEG and the biodegradable polymer used during formation of the nanoparticle, or as a coating of the PEG onto the nanoparticle surface. The PEG can be associated with the polymer by ionic, covalent, coordinate, hydrogen bonding, van der Waals, and other intermolecular forces, or be a simple blend. When PLGA, PLA, or PGA and PEG are utilized, e.g., as a triblock copolymer, the PEG or PEO often, though not necessarily, is the central block, and the polyester chains are at either end of the polymer. Studies have evaluated the effect of the length of a central PEG block (*J. Contrl. Rel., 24,* 81 (1993)) as well as the length of outer PLA blocks (*Macromolecules, 29*, 50, 57 (1996)) on water absorption and degradation of these copolymers. Kissel et al. have explored the synthesis of these tri-block materials, *in vitro* degradation, drug delivery, *in vitro* biocompatibility, and *in vivo* biocompatibility, as well as the microenvironment of PLA-PEO-PLA microparticles during degradation. The biocompatibility studies have shown that PLA-PEO-PLA polymers show very similar and minimal adverse tissue reactions. Drug delivery studies which compared *in vitro* delivery of bovine serum albumin from microparticles prepared from PLA-PEO-PLA and PLGA-PEO-PLGA polymers showed that the PLGA-containing polymers exhibited fairly continuous release profiles while PLA-containing polymers had two phases of release more typical of simple PLGA microparticles (*J. Contrl. Rel., 32*, 121 (1994)). Release studies of

macrophages in the circulatory system. Furthermore, smaller nanoparticles are known to cross into the cellular matrix, typically by endocytosis, and the size requirements of the nanoparticles is an important characteristic in transportability. Nanoparticles may enter a cell via the cellular caveloae, typically 20-60 nm openings that participate in receptor-mediated uptake processes, and via receptor-mediated endocytosis in clathrin-coated pits, typically in the range of 150-200 nm (see Unger et al., *supra*.) Furthermore a lining of cells in the bone functions as a marrow-blood barrier to limit the accessibility of exogenous large substances to the bone (Talmage, *Am. J. Anat., 129*, 467-76 (1970)). Consequently, an important aspect of the invention is the size and size distribution of the nanoparticles. In one embodiment, the nanoparticles of the invention have a diameter of about 10 nm to about 1000 nm. In a preferred embodiment, the nanoparticles have a diameter of about 50 to about 500 nm, more preferably from about 100 to about 400 nm, and even more preferably from about 100 to about 250 nm. The size distribution of the nanoparticles is also important since different sizes produce different release rates and different drug loading levels. The size range of the nanoparticles can be narrow, broad, or multimodal. The number of nanoparticles within a given size range can be greater than about 75%, greater than about 85%, greater than about 95%, or greater than about 99%. For example, if greater than 99% of the nanoparticles were within the range of 150-250 nm, then the distribution might be considered narrow, whereas greater than 75% of the nanoparticles within the range of 10-1000 nm might be considered broad. Alternatively, the size distribution of particles can be characterized by the relative polydispersity. Relative polydispersity is a value determined by the Coulter Nanosizer described below, and indicates the relative distribution around the median diameter. A relative polydispersity of 1 indicates a monodisperse sample, while increasing values indicate a broader distribution within the sample. The relative polydispersity can be less than about 5, preferably less than about 3, and more preferably less than about 2.

[0047] In another aspect of the invention, the composition comprises a bone targeting agent. By "bone targeting agent" is meant a chemical structure or ligand that has a high affinity for calcium ions in hydroxyapatite, the major constituent of bone. The composition of the invention can be targeted, in an embodiment, to calcium deposits in regions of the body other than bone, such as calcium deposits in the arteries, heart, kidney, or gall bladder. However, the bone targeting agent ideally selectively binds to bone tissue. A bone targeting agent of the invention is attracted to the bone tissue of the subject, preferably binds to the bone with a higher affinity than non-bone tissues, and remains bound for a certain length of time thereby delivering the composition to a bone environment. In other words, the bone targeting agent preferably binds to bone tissue with at least 2-fold greater affinity (e.g., at least 3-fold, at least 5-fold, at least 10-fold, or at least 25-fold greater affinity) than to a non-

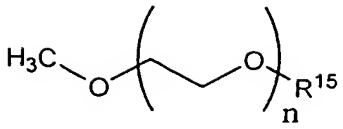
the radiation originating from the isotope bound to the osteoblastic bone metastases having some effect on the nearby metastatic tumor cells. Another clinically useful bone targeting system is DOTMP (the chemical structure of which is set forth in Figure 2), now in Phase III clinical trials (termed STR, skeletal targeted radiation) as the radioactive ^{166}Ho complex designed to deliver large doses of radiation selectively to the bone marrow for the treatment of multiple myeloma. It should be noted that the radioactive ^{166}Ho-DOTMP complex localizes in the skeletal system and irradiates the nearby bone marrow which houses the malignant myeloma cells. Like the ^{153}Sm-EDTMP system, the phosphonate that does not localize in the bone is cleared through the urine and out the body. See Figure 7 of Bayouth et al., *J. Nucl. Med., 36*, 730 (1995).

[0050] Preferably, the bone targeting agent is a polyphosphonic acid. Polyphosphonic acid has been demonstrated to successfully target biologically-active molecules to bone tissue. For example, conjugation (via isothiocyanato chemistry) of polyaminophosphonic acids, such as ABDTMP (the chemical structure of which is set forth in Figure 3), to growth factors (to stimulate bone formation) successfully resulted in the targeting of the growth factors to the bones of rats (see, for example, International Patent Publication WO 94/00145). Similarly, bone targeting agents have been coupled to proteins. For example bisphosphonates that were conjugated to human serum albumin successfully delivered the protein to bone *in vitro* (*Biotechnol. Prog., 16,* 258 (2000)) and *in vivo* (*Biotechnol. Prog., 16,* 1116 (2000)). The utility of bone targeting agents extends beyond delivery of proteins to bone and includes, for instance, small therapeutic molecules. A conjugate comprising a bone targeting bisphosphonate and an alkylating agent, such as BAD (the chemical structure of which is set forth in Figure 4), has been generated (see, for example, Wingen et al., *J. Cancer Res. Clin. Oncol., 111*, 209 (1986)). In this molecule, the alkylating agent is not specific in its interaction with its target (DNA), and, thus, there is no requirement for cleavage between the bisphosphonate (i.e., bone targeting agent) and the alkylating moiety. The bisphosphonate-alkylating agent demonstrated efficacy in a rat osteosarcoma model using BAD. Another series of studies have been performed using the antifolate antineoplastic agent methotrexate that has been covalently attached to bisphosphonates, designated MTX-BP and shown in Figure 5 (see, for example, Sturtz et al., *Eur. J. Med. Chem., 27*, 825 (1992); Sturtz et al., *Eur. J. Med. Chem., 28*, 899 (1993); and Hosain et al., *J. Nucl. Med., 37*, 105 (1996)). Using Tc-99m labeled MTX-BP, it was determined that around 15% of the injected dose was localized in the skeleton after 4 hours with about 61% of the dose being excreted (Hosain, *supra*). MTX-BP further demonstrated five times greater anticancer activity compared with methotrexate alone in animal models of transplanted osteosarcoma (*Sturtz* 1992, supra). Similar work has been described using the conjugate CF-BP, a carboxyfluorescein group with an appended bisphosphonate whose

[0054] It is envisioned that these bone targeting agents and other bone targeting agents can be attached through one of the heteroatoms or by chemical modification that installs an additional attachment point. For example, EDTMP can be connected to a linker by one of the phosphorous oxygens to create a phosphonate linkage, as illustrated in Figure 8 (see for example Vieira de Almedia et al., *Tetrahedron*, *55*, 12997-13010 (1999).) The phosphorous oxygen can also be alkylated as shown in Figure 9, where the R group can have, for example, a pendant amino group, to provide a secondary attachment point for ligation to, for example, an activated PEG. Other types of alkylation that could be utilized in the invention include but are not limited to examples similar to that involving DOTMP, as has been further described in Chavez et al., *Biomedical Imaging: Reporters, Dyes, & Instumentation*, Contag & Sevick-Muracia, Eds., Proc. SPIE, Vol. 3600, 99-106 (July, 1999), or as shown for other phosphonic acids further described in, for example, U.S. Patent 5,177,064, U.S. Patent 5,955,453, de Lombaert et al., *J Med. Chem.*, *37*, 498-511 (1994), and Iyer et al., *Tetrahedron Letters*, *30*(51), 7141-7144 (1989). Alternatively, for chemical modification, EDTMP can be, for example , modified to generate ABDTMP by installation of an aniline group (as further described in, for example, Figure 5 of International Patent Publication WO 94/00145). The aniline amine is then available to form, for example, an amide bond. DOMTP could be similarly modified, as outlined in Figure 10.

[0055] The terms "phosphonate, phosphate, and aminomethylenephosphonate" are meant to encompass the phosphonic acids, the phosphoric acids, and aminomethylenephosphonic acids, respectively, as well as any salts, hydrolyzable esters, and prodrugs of the phosphorous-based acids thereof. At the biological pH of 7.4 in the blood, or the more acidic pH around the bone, a certain portion of the phosphate or phosphonate of the bone targeting agent may be deprotonated and replaced with a counterion. Furthermore, the exchange of proton for calcium is an inherent event for the binding of the bone targeting agent to the hydroxyapatite in the invention. However, preparation and administration of the composition containing the bone targeting agent may or may not require complete protonation of the phosphorous acids therein. Therefore, the phosphonic acid, phosphoric acid, and aminomethylenephosphonic acid are drawn and utilized interchangeably with phosphate, phosphonate, and aminomethylenephosphonate. Biologically hydrolyzable esters of the phosphorus-based acids may also be utilized in the *in vivo* use of the bone targeting nanoparticles. Similarly, prodrugs of the phosphorous-based acids may also be utilized *in vivo* to mask the acidity of the composition during, for example, formulation and administration.

[0056] The nanoparticles can be prepared in any suitable manner. For example, the preparation methods for biodegradable microparticles known in the art can be used to prepare the nanoparticles of the invention. Most preparations are based on solvent



(VI)

wherein n is an integer from 2 to 2000, preferably from 10-1000, and more preferably from 30-200, and R^{15} a organic radical that contains an electrophilically activated leaving group. By "electrophilically activated leaving group" is meant a group that will be attacked by an incoming nucleophile, e.g., an amine or a alcohol, thereby forming a new covalent bond. Examples of R^{15} include but are not limited to epoxy groups, aldehydes, isocyanates, isothiocyanates, succinates, carbonates, propionates, butanoates, etc., such as succinimidyl glutarate, succinimidyl, succinimidyl succinamide, succinimidyl carbonate,, N-hydroxysuccinimidyl carbonate, propionaldehyde, succinimidyl propionate, succinimidyl butanoate, and the like. In a preferred embodiment, the R^{15} is a succinimidyl propionate or succinimidyl butanoate. An additional organic linkage may or may not be present between the activated PEG and the bone targeting agent, as demonstrated in the examples.

[0061] The bone targeting agents may be attached to the polyester by any suitable technique known in the art. In one embodiment, the bone targeting agent is attached to a polyester by reacting the bone targeting group with a polyester containing an activated ester end group, as are known in the art. (See, for example, Yoo, et al., *Pharmaceutical Research, 16*, 1114 (1999)). The activated ester end group may be present at either end of the polyester, for example, as shown in Figure 13 (the OR^* being a displaceable group.)

[0062] In one embodiment, biodegradable nanoparticles comprising an active agent, a biodegradable polymer, and a bone targeting agent can be prepared by a process comprising providing an organic phase, e.g., a suspension or a solution, with one or more of a biodegradable polymer, a PEG, an activated PEG, a bone targeting agent, a PEG-modified biodegradable polymer, a bone targeting agent- biodegradable polymer conjugate, a bone targeting agent-PEG conjugate, a bone targeting agent-PEG-modified biodegradable polymer conjugate, and an active agent therein, with the requirement that the organic phase contains at least one PEG, at least one biodegradable polymer, and at least one active agent, mixing the organic phase. The organic phase is mixed with an aqueous phase, e.g., a suspension or a solution, comprising water and a surface active agent. The organic solvent(s) are removed from the mixture while stirring, thereby recovering the resultant nanoparticles and optionally treating the nanoparticles with a bone targeting agent. The organic solvent or solvents can be any solvent used in the art, preferably a solvent selected from the group consisting of C_1-C_4 alcohols, C_2-C_6 esters, C_2-C_6 ethers, and C_1-C_6 organic acids. The surface active agent in the aqueous layer is any agent used in the art that aids in the formation of the nanoparticles, preferably bovine serum albumin, human serum albumin, or polyvinyl alcohol. The surface active agent may be in any concentration that provides for control of nanoparticle sizes. In one preferred embodiment the bovine serum

[0065] The invention also provides for a method of delivering an exogenous substance to a mammal. The exogenous substance is absorbed, adsorbed, encapsulated, or chemically bonded into a biodegradable nanoparticle that bears a bone targeting agent. The exogenous substance can be any known compound or mixture, and can modify a cellular response. Preferably the exogenous substance comprises one or more drugs, proteins, nucleic acids, or mixtures thereof. The exogenous substance can also be a therapeutic or biologically active agent.

[0066] In one embodiment, the biologically active or therapeutic agent can be a chemotherapeutic agent. Chemotherapeutic agents can include adriamycin, asparaginase, bleomycin, busulphan, cisplatin, carboplatin, carmustine, capecitabine, chlorambucil, cytarabine, cyclophosphamide, camptothecin, dacarbazine, dactinomycin, daunorubicin, dexrazoxane, docetaxel, doxorubicin, esperamicin, etoposide, floxuridine, fludarabine, fluorouracil, gemcitabine, hydroxyurea, idarubicin, ifosfamide, irinotecan, lomustine, mechlorethamine, mercaptopurine, meplhalan, methotrexate, mitomycin, mitotane, mitoxantrone, nitrosurea, paclitaxel, pamidronate, pentostatin, plicamycin, procarbazine, rituximab, streptozocin, tauromustine, teniposide, thioguanine, thiotepa, Vinca alkaloids, vinblastine, vincristine, vinorelbine, paclitaxel, transplatinum, 5-fluorouracil, and the like.

[0067] The modification of cellular response can be temporary or a permanent inhibition, e.g., of p53. The gene for p53 is well known and has been studied extensively. The p53 protein is a key player in the cellular stress response mechanism. For example, in response to DNA damage the tumor suppressor protein p53 shuts down cell division or causes the cell to undergo apoptosis (programmed cell death). In this manner p53 can serve to stop tumor formation by stopping cells that have incurred malignant mutation from growing. The p53 gene is susceptible to damage and if damaged it can contribute to genetic instability and ultimately possible tumor formation. It is thought that roughly half of all cancers (including skin, breast, and colon cancers) possess mutant inactive p53 genes. On the other hand, p53 imparts sensitivity to normal tissue subjected to genotoxic stress such as radiation therapy or chemotherapy. In particular, damage to the lymphoid, hematopoietic system, intestinal epithelium, and even hair follicles contribute to collateral damage when undergoing cancer therapies and serve to limit the maximum tolerated doses of treatment.

[0068] The modification of a cellular response to impart protective activity (e.g., p53 inhibition) need not be in response to exogenous, environmental stimuli. Many processes in the body can result in cell damage, which can be inhibited by administration of the composition of the invention. For example, ischemia and ischemia/reperfusion injury can be minimized by a inhibiting cell death. Ischemia is often caused by an interruption of the supply of oxygenated blood, such as that caused by a vascular occlusion. Vascular occlusions can be caused by arteriosclerosis, trauma, surgical procedures, disease, and/or

butynyland the like. The term "alkyl," "alkenyl," and "alkynyl" is also meant to include cycloalkyl, cycloalkenyl, and cycloalkynyl moieties (e.g., "C_1-C_6 alkyl" encompasses cycloalkyl, C_3-C_6 alkenyl emcompasses cycloalkenyl with rings of 3 to 6 carbons, etc.)

[0073] By "aromatic" is meant a monocyclic or polycyclic set of unsaturated carbons, e.g., phenyl. Similarly, "heteroaromatic" is a monocyclic or polycyclic set of carbons wherein one or more carbons is replaced with a nitrogen, oxygen, or sulfur atom. Examples include, but are not limited to, furyl, pyridyl, pyramidyl, quinolyl, thienyl, and thiazyl groups. It is understood that the term aromatic applies to cyclic substituents that are planar and comprise $4n+2\pi$ electrons, according to Hückel's Rule.

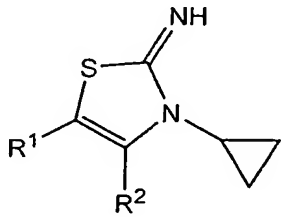
[0074] By "alkoxy" is meant an –OR group, wherein R is alkyl or aryl.

[0075] By "amino" is meant an $-NH_2$ group. By "alkylamino" is meant an $-NH_2$ substituted with one or two C_1 -C_6 alkyl or aryl groups, e.g., monoalkyl and dialkylamino. Examples include, but are not limited to, amino, methylamino, dimethylamino, diethylamino, methylethylamino, or phenylamino.

[0076] By "alkylthio" is meant an organic radical derived from an open, straight or branched hydrocarbon chain wherein the terminus of the organic radical terminates in a -SH group (thiol group).

[0077] By "acyl" is meant a carbonyl-R group, i.e., –C(=O)-R, wherein the carbonyl is bound to an alkyl group and a heteroatom.

[0078] The biologically active compound can be a compound of Formula I, wherein m is 0, n is 2, and R^3 is a one-carbon alkyl such that the three-carbon chain forms a cyclopropyl group. In other words, the biologically active compound is a compound of Formula II:



(II)

where R^1 and R^2 taken together form an aliphatic or aromatic carbocyclic 5- to 8-membered ring optionally substituted with one or more straight or branched C_1-C_6 alkyl, C_1-C_6 alkoxy, fluoro, chloro, bromo, nitro, amino, C_1-C_6 alkylamino, and/or C_4-C_{14} aromatic or heteroaromatic moieties.

[0079] The biologically active compound can be a compound of Formula I or Formula II, wherein R^1 and R^2 taken together form a 5- or 6-membered aliphatic carbocyclic ring. The 5- or 6-membered aliphatic carbocyclic ring optionally is substituted with one or more C_1-C_6 alkyl groups.

wherein R^1 and R^2 taken together form an aliphatic or aromatic carbocyclic 5- to 8-membered ring, optionally substituted with one or more straight or branched C_1-C_6 alkyl, C_1-C_6 alkoxy, fluoro, chloro, bromo, nitro, amino, C_1-C_6 alkylamino, and/or C_4-C_{14} aromatic or heteroaromatic moieties. R^3 is selected from the group consisting of a C_1-C_6 alkyl group, a C_1-C_6 alkoxy group, and a phenyl group, wherein the alkyl group, the alkoxy group, or the phenyl group is optionally substituted with one or more straight or branched C_1-C_6 alkyl, C_1-C_6 alkoxy, hydroxy, fluoro, chloro, bromo, nitro, amino, C_1-C_6 alkylamino, and/or C_4-C_{14} aromatic or heteroaromatic moieties. Preferably, the biologically active compound is 2-p-Tolyl-5,6,7,8-tetrahydro-benzo[d]imidazo[2,1-b]thiazole (i.e., pifithrin-β, shown in Figure 12).

[0082] Pifithrin-α was recently disclosed during work based on the hypothesis that if one could block p53 protein on a temporary basis in an animal with p53 deficient tumors then one could prevent the p53 initiated cell death in the normal tissues and hence prevent many of the side effects associated with chemotherapy and/or radiation treatments. (See U.S. Patent 6,593,353 and U.S. Published Patent Application 2003/0176318) A 10 micromolar concentration of pifithrin-α inhibited apoptosis induced by doxorubicin, etoposide, Taxol, cytosine arabinoside, UV light, and gamma radiation in C8 (mouse embryo fibroblasts transformed with Ela+ras) cells. To be effective, pifithrin-α needed to be present during or immediately (less than 3 hours) after exposure to UV, for example, in order to provide the protective effect. Pretreatment with removal before the stress-inducing event provide no significant protection. Pifithrin-α was also tested in two different strains of mice with the pifithrin-α being administered as a single intraperitoneal injection (2.2 mg/kg of body weight). Remarkably, this compound completely rescued both types of mice from 60% killing doses of gamma radiation (8 Gy for C57BL strain and 6 Gy for Balb/c strain). Additionally the treated animals experienced less weight-loss than controls. Importantly, in p53-null mice controls treated with radiation the pifithrin-α injections had no protective effect. Lastly, inhibition of p53 could potentially lead to tumor formation yet no tumors or pathological lesions were found in the pifithrin-α treated, gamma-irradiated survivors even after 7 months post-irradiation.

[0083] One concern with systemic administration of pifithrin-α or pifithrin-β is the potential for side effects. These types of heterocyclic compounds have known biological activities including interaction with alkaline phosphatase, glutamate transmission in epilepsy, and influencing multidrug resistance via P-glycoproteins. In addition, systemic administration of a temporary p53 inhibitor, e.g., pifithrin-α or pifithrin-β, during concurrent chemotherapy or radiation treatment would prevent cell death in the cancer cells. Thus, one major advantage of the present invention is the targeting of such molecules to the

weight. The biodegradable polymer can be present in 1-99%, preferably 10-90% and more preferable 25-85% by weight. The PEG may be present in 0.1-50%, preferably 1-40% and more preferably 5-25% by weight. The bone targeting agent may be present in from 0.1-50%, preferably 0.5-25% and more preferably 1-10% by weight.

[0089] The composition of the invention may be formulated in various manners, especially for administration to a mammal in, for example, therapeutic and prophylactic treatment methods. The composition for use in the inventive method comprises one or more compounds described herein and a physiologically-acceptable (e.g., pharmaceutically-acceptable) carrier. Pharmaceutically-acceptable carriers are well-known to those who are skilled in the art, as are suitable methods of administration of such compositions to a mammal. The choice of carrier will be determined in part by the particular compound within the composition, as well as by the particular method used to administer the composition. Likewise, various routes of administering a composition to a mammal are available. Although more than one route may be available, a particular route of administration may provide a more immediate and more effective response in the mammal than another route.

[0090] Ideally, the composition of the invention (e.g., a bone targeting biodegradable nanoparticle containing a therapeutic agent) is administered parenterally (e.g., subcutaneous, intramuscular, intravascular, intraspinal, intrasternal, intravenous, intrathecal, or intraarterial administration). Formulations suitable for parenteral administration are well known in the art, and include aqueous and non-aqueous, isotonic sterile injection solutions, which may contain anti-oxidants, buffers, bacteriostats, and solutes that render the formulation isotonic with the blood of the mammal, and aqueous and non-aqueous sterile suspensions that may include suspending agents, solubilizers, thickening agents, stabilizers, and preservatives. Parenteral formulations can be presented in unit-dose or multi-dose sealed containers, such as ampules and vials, and may be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example, water, for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules, and tablets.

[0091] In one embodiment, a composition or nanoparticles of the invention is administered directly to the area surrounding bone. While such procedures are invasive, direct administration to bone or bone marrow can provide a more immediate effect than, for instance, intravenous administration. A surgical procedure similar to that for aspirating bone marrow can be performed to administer the inventive composition directly to bone marrow. At least a portion of the inventive composition remains attached to the bone tissue via the bone targeting agent, which creates a sustained release mechanism of the biologically active agent to the bone marrow.

[0096] The inventive composition can be packaged in unit dosage form, i.e., physically discrete units suitable as unitary dosages for a mammal, each unit containing a predetermined quantity of the composition or nanoparticles calculated in an amount sufficient to produce the desired level of cellular response modification in association with a pharmaceutically acceptable diluent, carrier, or vehicle. Unit dosage forms can be incorporated into a kit, wherein the composition of the invention is provided in combination with a physiologically-acceptable carrier and instructions for administration to a mammal.

[0097] The following examples further illustrate the invention but, of course, should not be construed as in any way limiting its scope.

[0098] Materials and Methods: HPLC analysis was performed on a Shimadzu LCMS-2010 and employed a flow rate of 3 ml/min and a starting B concentration of 5%. The B solvent was linearly ramped to 95% concentration at 5.0 minutes, held at 95% until 6.0 minutes, then linearly ramped back down to 5% at 6.5 minutes, where it remains until the end of the run at 7.5 minutes. In addition to an electrospray mass spectrometer, the LC detection consisted of 3 channels: UV absorbance at 254 nm, UV absorbance at 214 nm, and evaporative light scattering (Alltech ELSD 2000). The evaporative light scattering detector was run at 50 C with a nitrogen flow of 1.5 liters per minute. The CDL and block temperatures of the Shimadzu LCMS-2010 were both 300°C, and the nitrogen nebulizer gas flow was 4.5 L./min. Positive and negative mass spectra were detected from 50 to 2000 m/z. The column was a YMC CombiScreen ODS-AQ, S-5μ particle size, 50 mm long with a 4.6 mm I.D. Mobile phase A was made using HPLC grade B&J water with 0.1% (v/v) HOAc added and mobile phase B was HPLC grade B&J acetonitrile with 0.1% (v/v) HOAc added. This system gives a retention time of 1.50 to 1.60 minutes for a standard commercially available material (4-hydroxyphenylacetic acid; Aldrich Catalog H5000-4; m.p. 149-151 C) used as a reference standard.

[0099] Gradient Preparative HPLC was performed on a Shimadzu system composed of two LC-8A pumps connected to a SIL-10A autosampler and eluting over a reverse phase column (YMC, cat CCAQSOSO52OWT; ODS-AQ CombiPrep, 20 mm X 50 mm) and then passing through an MRA variable volume splitter; the smaller stream was then made up to 3 mL/minute using a LC-10ADVP make-up pump (MeOH) and the eluent passed through a variable two channel wavelength UV detector and then split roughly 6:1 to an evaporative light scattering detector (run at 50 C with a nitrogen flow of 1.5 liters per minute) and a Shimadzu 2010 Mass detector; the larger stream from the MRA splitter then flowed to a Gilson 215 liquid handler serving as a fraction collector triggered by mass, UV absorbance, or ELS peak size. Different gradients were run always starting with the more aqueous solvent A and ramping up to various concentrations of B. Mobile phase A was

EXAMPLE 3

[0104] This example illustrates a method for preparing a PEG-modified bone targeting agent.

[0105] A solution of 500 mg 4-[(N-Boc)aminomethyl]aniline (S in Figure 15) in 10 mL dioxane was treated with paraformaldehye (400 mol%, 270 mg) and trimethylphosphite (400 mol%, 1.12 g). The mixture was heated to 95°C overnight. More paraformaldehyde (270 mg) and trimethylphosphite (1.12 g) were added and the mixture was heated at 95°C overnight again. The solution was cooled, taken up in chloroform (20 mL) and washed with saturated sodium chloride (20 mL) and water (20 mL). The organics were dried over sodium sulfate and the solvent and excess trimethylphosphite removed via rotary evaporation at 80°C to provide 1.723 g of a clear oil. The presence of the diphosphonate (**T**) was confirmed by electrospray HPLC-MS showing a retention time of t_R = 2.9 minutes and a mass of 467 *m/z* [M+H]+ and 489 *m/z* [M+Na]+ found for the desired mass [M=$C_{18}H_{32}N_2O_8P_2$].

[0106] A solution of 870 mg diphosphate (**T**) in 10 mL dichloromethane was treated with bromotrimethylsilane (690 mol%, 1.97 g). The solution was stirred overnight. Methanol (10 mL) was added and the solution was stirred 15 min and then concentrated to provide 1.12 g of an orange oil. The presence of the diphosphonic acid (**U**) was confirmed by electrospray LC-MS. The retention time using this gradient was found to be t_R = 0.85 minutes and the mass spec for the desired product [M=$C_9H_{16}N_2O_6P_2$] found at the expected *m/z* 309 $[M-H]^-$ operating in the negative mode.

[0107] A 50 mg portion of the diphosphonic acid (**U**) was solubilized in 200 uL of water and adjusted to pH= 8.1 using NaOH and then treated with 100 mg (0.125 equivalents) of PEG-SPA (Shearwater; 5,000 molecular weight) and 100μL of water, then allowed to stir for 24 hours. Analysis by HPLC showed that the starting PEG-SPA peak had largely disappeared (retention time 2.97 minutes) and was replaced by a UV-absorbing peak at about 3.75 minutes. The sample was then purified on prep HPLC to give 44.6 mg of the bone targeting aminodiphosphonic acid (**V**) coupled to a 5,000 molecular weight PEG) having a retention time of 3.75 minutes and exhibiting UV activity at 254 nm and exhibiting a mass spectrum supportive of a polymeric structure.

EXAMPLE 4

[0108] This example illustrates a method for preparing a PEG-Fluorescein complex.

[0109] A solution of 210 mg *p*-xylylenediamine (W in Figure 16) in 6 mL methanol was treated with a fluorescein isothiocynate (X, FTIC Isomer 1 – Calbiochem, catalog number 34321) in small portions over 1 hr. During the addition, a red-brown solid was produced. The mixture was stirred overnight, filtered, and washed with methanol (2 mL).

starting activated ester to the desired product (**AB**) with a retention time of 1.608 minutes and the expected mass spectrum ([M+H]+ =294 for C15H23N3O3-H+).

[0115] A solution of 872 mg of the amine (**AB**) in 10 mL dioxane was treated with paraformaldehyde (535 mg) and trimethylphosphite (2.21 g). The mixture was heated at 100°C overnight and then the solvent removed by rotary evaporation at 80°C to give a brown solid. Chloroform (25 mL) was added and the solution was washed with water (15 mL). The organics were dried over sodium sulfate and the solvent removed to provide 241 mg of a yellow semi-solid. This was purified via LC to provide 58.8 mg of diphosphonate ester (**AC**) material. The compound was confirmed by electrospray LC-MS using method A; t_R = 2.6 min. MS [M=$C_{21}H_{37}N_3O_9P_2$] m/z 538 (MH^+), 560 (MNa^+).

[0116] A solution of 54.6 mg of diphosphonate (**AC**) in 1 mL dichloromethane was treated with bromotrimethylsilane (156 mg). The mixture was stirred overnight. Ethanol (0.5 mL) and water (3 drops) were added and it was stirred 1 hr and then the volatiles were removed and the material dried under vacuum. This was taken up in water (1 mL) and lyophilized to provide 59 mg of a tan solid. The presence of the free amine diphosphonic acid (**AD**) was confirmed by electrospray LC-MS using method B; t_R = 0.4 min. MS [M=$C_{12}H_{21}N_3O_7P_2$] m/z 380 ($M-H^-$), 382 (MH^+), 404 (MNa^+)

[0117] The amine diphosphonate can be converted to a PEG-modified bone targeting agent by treating 50 mg with 650 mg of mPEG-SPA in water, then freeze-drying to isolate the PEG-aminodisphosphonic acid bone targeting agent.

EXAMPLE 6

[0118] This example illustrates a method for preparing a tetraphosphonic acid bone targeting agent.

[0119] To a solution of 2.1 g tris-(2-aminoethyl)amine (see Figure 17) in 20 mL tetrahydrofuran was added dropwise a solution of 1.0 g of the activated ester (**AA**) in 20 mL tetrahydrofuran over a period of 40 minutes. The mixture was stirred overnight resulting in a precipitate that was filtered and concentrated via rotary evaporation to provide 2.10 g of a yellow oil. The presence of the diamine (**AE**) was confirmed by electrospray LC-MS using method A; t_R = 1.4 min. MS [M=$C_{19}H_{33}N_5O_3$] m/z 380 (MH^+), 402 (MNa^+).

[0120] A solution of 2.08 g diamine (**AE**) in dioxane (20 mL) was treated with paraformaldehyde (1.50 g) and dimethylphosphite (6.85 g). The mixture was stirred at 90°C overnight and the solvent removed via rotary evaporation at 70°C. Dichloromethane (50 mL) was added and it was washed with saturated sodium chloride (25 mL) and water (25 mL). The organics were dried over sodium sulfate and the solvent removed. The residue was purified via LC to provide 123.8 mg of a yellow oil. The presence of the

concentrations of pifithrin α or pifithrin β of 100, 500, and 1000 μM was used for quantitation using the 254 nm absorbance for the cell protection factor peak.

[0126] The experimental details and analysis for the nanoparticle preparations are set forth in Table 1. In a typical procedure, the organic solution (shown in column 2 of Table 1) was sonicated for 30-120 seconds, then added to the aqueous layer (shown in column 3) contained within a 20 ml scintillation vial. Deionized water was used to prepare the aqueous solutions and water washes. This solution was sonicated for an additional 60 seconds, then transferred to a 125 ml Erlenmeyer flask and set to stir at 250 to 500 rpm under vacuum for 45 minutes (shown in column 4, "Experimental Conditions"). Vacuum was typically achieved at about 3 to 10 inches of mercury. After 45 minutes, the contents were poured into a centrifuge tube and the flask rinsed with 10 ml of water. The particles were spun down at 18000 rpm for 10 minutes and the supernatant pipetted out. A 10 ml wash solution of a 1 mg/ml BSA solution was added to the centrifuge tube, the sample was sonicated until the particles were completely resuspended, the sample was spun down at 18000 rpm for 10 to 15 minutes, and the supernatant was removed by pipet. This wash/spin cycle was repeated a total of three times. After the third cycle, the supernatant was removed, the particles were frozen overnight, and placed on a freeze dryer for 24 hours. Analytical results are reported in Table 1 for each preparation.

EXAMPLE 8

[0127] This example illustrates the binding of nanoparticles in accordance with an embodiment of the invention to hydroxyapatite.

[0128] Nanoparticles selected from examples 7.9, 7.10, 7.11, 7.12, 7.13, and 7.31 above were assayed for hydroxyapatite binding. For each sample, a nominal 7.5 mg of each nanoparticle preparation was weighed and 1.5 ml Tris-buffered saline (50 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol and 150 mM NaCl, pH 7.4) was added, for a concentration 5.0 mg/ml. The samples were then subjected to ultrasonication for one minute to form a uniform suspension of particles.

[0129] A 10.0 mg/ml suspension of hydroxyapatite was prepared by weighing 80 μm hydroxyapatite particles (Bio-Rad MacroPrep Ceramic Hydroxyapatite Type I 80 μm - Catalog No. 185-8000) and diluting with Tris-buffered saline. The hydroxyapatite particles settle quickly, so the suspension is stirred on a Thermolyne magnetic stirplate at the slowest possible speed to achieve uniformity, and sampling by pipette is performed quickly to minimize fragmentation of the hydroxyapatite particles.

Table 1 (cont.)

7.6	100 mg PLGA in 2.0 ml acetone 1 ml of 10 mg/ml pifithrin-β in methanol	10 ml of 1 mg/ml BSA in water	Sonicate 30 sec Stir at 500 rpm under vacuum	90.4	234	4.7	NA	5
7.7	100 mg PLGA in 2.5 ml acetone 10 mg pifithrin-β 1.5 ml methanol	10 ml of 1 mg/ml BSA in water	Sonicate 30 sec Stir at 250 rpm under vacuum	92.7	306	12.5	NA	5
7.8	100 mg PLGA in 3.5 ml acetone 10 mg pifithrin-β 1.5 ml methanol	10 ml of 10 mg/ml BSA in water	Sonicate 30 sec Stir at 500 rpm under vacuum	106.3	225	8.9	NA	3
7.9	100 mg PLGA in 2.0 ml acetone 10 mg PEG-alendronate complex 1 ml of 10 mg/ml pifithrin-β in methanol	10 ml of 10 mg/ml BSA in water 10 ml water	Sonicate 60 sec Stir at 500 rpm under vacuum	92.1	208	10.9	NA	2
7.10	100 mg PLGA in 2.0 ml acetone 10 mg PEG-alendronate complex 20 mg pifithrin-β	10 ml of 10 mg/ml BSA in water 10 ml water	Sonicate 120 sec Stir at 500 rpm under vacuum	103.3	301	9.6	NA	4
7.11	100 mg PLGA in 4.0 ml acetone 10 mg PEG-alendronate complex 2 ml of 10 mg/ml pifithrin-β in methanol	10 ml of 10 mg/ml BSA in water 10 ml water	Sonicate 60 sec Stir at 500 rpm under vacuum	121.3	187	16.2	NA	4

Table 1 (cont.)

7.17	100 mg PLGA in 2.0 ml acetone 10 mg PEG-alendronate complex 10 mg pifithrin-β 1 ml methanol	10 ml of 10 mg/ml BSA in water 10 ml water	Sonicate 60 sec Stir at 500 rpm under vacuum	101.4	238	2.9	NA	2
7.18	100 mg PLGA in 2.0 ml acetone 15 mg PEG-alendronate complex 10 mg pifithrin-β 1 ml methanol	10 ml of 10 mg/ml BSA in water 10 ml water	Sonicate 60 sec Stir at 500 rpm under vacuum	101.4	244	4.2	NA	3
7.19	100 mg PLGA in 2.0 ml acetone 20 mg PEG-SPA 1 ml of 10 mg/ml pifithrin-β	10 ml of 10 mg/ml BSA in water 2.7 ml alendronate	Sonicate 60 sec Stir at 500 rpm under vacuum	104.2	-	5.3	NA	-
7.20	100 mg PLGA in 2.0 ml acetone 10 mg pifithrin-α 1 ml methanol	10 ml of 10 mg/ml BSA in water 10 ml water	Sonicate 60 sec Stir at 500 rpm under vacuum	102	248	3.9	0.3	5
7.21	100 mg PLGA in 2.0 ml acetone 20 mg pifithrin-α 1 ml methanol	10 ml of 10 mg/ml BSA in water 10 ml water	Sonicate 60 sec Stir at 500 rpm under vacuum	107.7	298	7.2	0.5	6
7.22	100 mg PLGA in 2.0 ml acetone 10 mg PEG-SPA 10 mg pifithrin-α 1 ml methanol	10 ml of 10 mg/ml BSA in water 10 ml water	Sonicate 60 sec Stir at 500 rpm under vacuum	103.9	247	6.4	0.3	5

Table 1 (cont.)

7.28	100 mg PLGA in 4.0 ml acetone 25 mg PEG-Alendronate complex 10 mg pifithrin-β 1 ml methanol	10 ml of 10 mg/ml BSA in water 10 ml water	Sonicate 60 sec Stir at 500 rpm under vacuum	100.9	256	13.6	NA	3
7.29	100 mg PLGA in 2.0 ml acetone 20 mg PEG-Alendronate complex 10 mg pifithrin-β 1 ml methanol	10 ml of 10 mg/ml BSA in water 10 ml water	Sonicate 60 sec Stir at 500 rpm under vacuum	102.3	330	3.9	0.2	7
7.30	100 mg PLGA in 3.0 ml acetone 10 mg PEG-aminomethylaniline bisphosphonate (V) 10 mg pifithrin-β 1 ml methanol	10 ml of 10 mg/ml BSA in water 10 ml water	Sonicate 60 sec Stir at 500 rpm under vacuum	101.8	300	5.9	NA	6
7.31	100 mg PLGA in 4.0 ml acetone 12.3 mg of PEG-Alendronate complex 1 ml of 10 mg/ml pifithrin-β in methanol	10 ml of 10 mg/ml BSA in water 10 ml water	Sonicate 60 sec Stir at 500 rpm under vacuum	-	-	-	NA	-

*NA – not applicable

microscopy (SEM.) The SEM images are shown in Figures 18 to 21. In each set of figures, the first figure shows the entire particle and the second figure shows the surface of the particle. Note the scale in the lower right corner of each photo.

[0135] Figure 18A and 18B show the BioRad hydroxyapatite particles as received. Figures 19A and 19B shows the surface of the hydroxyapatite particles after exposure to nanoparticles prepared in Example 7.26. The nanoparticle lacked any bone targeting groups in this control, and produced a hydroxyapatite surface without nanoparticles. In comparison, Figures 20A and 20B after exposure to bone targeting nanoparticles in Example 7.27, and Figures 21A and 21B after exposure to bone targeting nanoparticles from Example 7.30, clearly demonstrated the binding of the bone targeting nanoparticles to the hydroxyapatite surface.

EXAMPLE 10

[0136] This example illustrates a method for preparing nanoparticles containing pifithrin-α or pifithrin-β.

[0137] Biodegradable nanoparticles containing pifithrin-α and pifithrin-β are prepared by dissolving appropriate amounts of PLA, PGA, and PLGA in acetone or ethyl acetate and subsequently adding appropriate amounts of drug, which are then dissolved in the polymer/solvent solution. The release behavior of the nanoparticles is altered by changing the amount of solvent, the amount of drug, the ratio of PLA to PGA, the amount of reactive PEG, and the physical conditions during the nanoparticles preparation such as mixing speed and temperature.

[0138] Specifically, to give nanoparticle formulations with sizes under 500 nm, 100 mg of PLA is dissolved in 1.5-3.0 ml of ethyl acetate. The biodegradable polymer used is commercially available poly(lactic acid), acid end-capped, 10-20,000 molecular weight. Concurrently, 10-30 mg of mPEG-SPA, molecular weight 5,000, from Nektar, formerly Shearwater Polymers, is dissolved in 1 ml of methanol, which is added to the polymer solution and mixed. After the polymer dissolves, 5-15 mg of pifithrin-α or pifithrin-β is added to the polymer solution and allowed to stand with moderate swirling until the drug dissolves. The drug/polymer mixture is then poured into 50 ml of a 10 mg/ml aqueous bovine serum albumin or 1.0 % poly(vinyl alcohol) solution and stirred for approximately 30 minutes under moderate vacuum at 500 RPM to allow extraction and evaporation of the organic solvents.

EXAMPLE 11

[0139] This example illustrates a method for preparing nanoparticles containing PLA and a PEG-modified bone targeting agent.

nanoparticle reactive groups) is added to the stirring mixture and the reaction is allowed to proceed for 1 hour. The supernatant is sampled to determine the course of the reaction progress. These techniques have been used to successfully attach fibrinogen and the Her2 antibody (Herceptin) to SSA-PEG and this method is a modification of published techniques (Hermanson, G. T., Bioconjugates, Academic Press, San Diego (1997)).

EXAMPLE 15

[0147] This example illustrates a method for preparing an activated ester of PGLA and subsequent preparation of a bone targeting agent PLGA conjugate (AI) or PEG modified PLGA (AJ) in Figure 23.

[0148] Polylactic-polyglycolic acid (PLGA) was obtained from Alkermes as Medisorb polymer catalog 5050DL2A (lot 9007-394) which had a molecular weight of 11 kD, polydispersity of 1.7, mole ratio of D,L-lactide of 53% and glycolide ratio of 47% with Tg of 41.3 C and inherent viscosity of 0.17 dL/g and had no endcap (i.e. the polymer has an alcohol terminus at one end and a carboxylic acid at the other end). Various other polymers with different lactide to glycolide ratio as well as various viscosities and various ester endcaps for the carboxylic acid group are commercially available from Alkermes and are suitable to substitute in place of the particular PLGA used herein. This procedure was based on H.S. Yoo et. al. in Pharmaceutical Research Vol 16(7), 1999 pp1114-1118.

[0149] A solution of 10.0 g of PLGA dissolved in 100 mL of methylene chloride was treated with 720 mg of p-nitrophenylchloro formate(Aldrich) and cooled to 0 C in an ice bath. The solution was treated with 480 uL of pyridine in a dropwise fashion and then allowed to warm to room temperature over three hours. The reaction mixture was transferred to a separatory funnel and was then washed with 1N HCl and then with brine. The organic layer was dried over sodium sulfate and then rotoevaporated to yield 10.53 g of the PGLA-nitrophenylformate (AH) as a white solid foam. This material gave a single peak with a retention time on HPLC-MS of 5.517 minutes and the mass spectrum of this peak was indicative of a polymeric structure. (See Figure 23.)

[0150] The PGLA-nitrophenylformate can be subsequently reacted with a bone targeting agent such as alendronate to produce a bone targeting PGLA (AI), or other amine-bearing bone targeting agents such as those shown in Figure 7, or Figure 8, or U of Figure 15 or AD or AG of Figure 17 or ABDTMP of Figure 3, etc. The PGLA – nitrophenylformate can alternatively be reacted with mPEG-NH_2 to produce mPEG-PLGA (AJ).

EXAMPLE 17

[0153] This example illustrates a method for preparing aminotetraphosphonic acid-PEG-PGLA (**AP**) in Figure 25.

[0154] A 36 mg (55 uMole) portion of amine tetraphosphonic acid (**AG** – see Figure 25) prepared in Example 6 was dissolved in 1 mL of 1M sodium bicarbonate to give a solution with a pH of about 8-9 by pH paper. To this was added 170 mg of Boc-NH-PEG-NHS (Nektar Therapeutics, catalog number 4M530F02 , 3100kD molecular weight) which went into solution with vigorous stirring. After 11 hours at room temperature the reaction was analyzed by HPLC-MS which showed that >60% of the starting PEG had been converted to a broad new peak (retention time 4.03 minutes) possessing UV activity at 254 nm characteristic of covalently incorporating the phenyl group of the bone targeting agent onto the polymer. The mass spectra of this new peak also indicated a polymeric structure. This solution was assigned to Boc-NH-PEG-aminotetraphosphonic acid (**AN**). All of this material was purified by preparative HPLC-MS to give 47.2 mg of white solid.

[0155] A 47.2 mg portion of the Boc-NH-PEG-aminotetraphosphonic acid was dissolved in 1 mL of trifluoroacetic acid and allowed to stand at room temperature for 30 minutes. An aliquot was removed and blown down with argon and dissolved in acetonitrile and analyzed by HPLC-MS. This analysis showed that all of the starting material had been converted to a new peak with retention time of 2.985 minutes showing UV activity and mass spectra consistent with a polymer. The reaction solution was rotoevaporated to give 52.7 mg of the whites solid NH_2-PEG-aminotetraphosphonic acid (**AO**).

[0156] A 40 mg portion of NH_2-PEG-aminotetraphosphonic acid was dissolved in 150 µL of dry acetonitrile and treated with 102 mg of PGLA-nitrophenylformate (**AH**) to give a clear solution. This solution was treated with 200 µL of triethylamine and the solution allowed to stand at room temperature. After 15 hours an aliquot was analyzed by HPLC-MS and showed complete disappearance of the starting material and showed slight UV absorption associated with the PLGA peak (retention time 5.356 minutes, broad). The desired reaction was further confirmed by the appearance of para-nitrophenol indicating conjugation had occurred. The reaction solution was rotoevaporated to give 147 mg of sticky yellow solid assigned PGLA-PEG-aminotetraphosphonic acid (**AP**).

EXAMPLE 18

[0157] This example illustrates a preparation of bone targeting nanoparticles composed of PLA and a bone targeting PEG.

[0158] To a solution of 1 ml ethyl acetate and 1 ml methanol is added 100 mgs of PLA, 50 mgs of a PEG conjugate of the amine tetraphosphonic acid (**AG**) from Example 7, and 10 mgs of pifithrin β, and the solution sonicated for 90 seconds. The resulting solution is

aqueous solution containing 3 mg of amine tetraphosphonic acid (**AG**) from Example 6, and stirred under moderate vacuum at 500 rpm for 45 minutes. The resultant nanoparticles are isolated from the aqueous solution by centrifuge and lyophilization.

EXAMPLE 22

[0165] This example illustrates a method for preparing bone targeting nanoparticles composed of a PEG-modified PLA, PLGA, and a bone targeting PEG.

[0166] To a solution of 1 ml acetone and 1 ml methanol is added 50 mgs of a PLA-PEG conjugate, 50 mgs of PGLA from Alkermes as Medisorb polymer catalog 5050DL2A, and 50 mgs mPEG-SPA. This mixture is sonicated for 60 seconds to completely dissolve the reagents, then 30 mgs of pifithrin β is added and allowed to dissolve. The resulting solution is then poured into 50 ml of 1.0 % poly(vinyl alcohol) aqueous solution containing 6 mg of amine tetraphosphonic acid (**AG**) from Example 6, and stirred under moderate vacuum at 500 rpm for 45 minutes. The resultant nanoparticles are isolated from the aqueous solution by centrifuge and lyophilization.

EXAMPLE 23

[0167] This example demonstrates methods for characterizing nanoparticles.

[0168] The nanoparticle size distribution is analyzed using a Coulter Nanosizer, which reports a median diameter and a relative polydispersity. (A polydispersity of 1 represents a monodisperse sample.) The Coulter Nanosizer is calibrated with 200 nm latex spheres (Polyscience, Warrington, PA.) In some instances, aggregation of the sample may be observed, and can produce a median particle diameter of greater than 1 micron and a relative polydispersity of above about 9.

[0169] The specific methods by which the particles are prepared can be modified in order to maximize the percentage of particles that are smaller than 500 nm, preferably less than 300 nm, in diameter. In addition, particles are tested using hydrophobic interaction chromatography to evaluate the relative amount of PEG at the particle surface. Hydrophobic interaction chromatography (HIC) is used to detect the presence of PEG at the particle surface. Samples are prepared by dispersing particles in saline at approximately 2 mg/ml, filtering the solution with 1.2 μm glass fiber filter paper, and injecting 1 ml of this solution onto the HIC column. The opacity of subsequent saline washes through the column is measured at 400 nm on a UV-Vis spectrophotometer. Specifically, a 1 ml-capacity butyl or phenyl sepharose column is charged with the particle solution. Saline is pumped through the column at 0.8 ml/min and the effluent is collected continuously in 5 minute intervals for 10 min. Then 1 ml of Triton X (0.01% v/v in phosphate buffered saline) is used as a first wash to remove any slightly bound particles. Another 5 minute

relationship between the stealthiness of nanoparticles and having enough bone targeting agent to direct the nanoparticle to the bone surface.

EXAMPLE 25

[0174] This example illustrates a method for measuring the degradation of the biodegradable nanoparticle.

[0175] Only nanoparticles with bone targeting agents attached that approach ten times of the bone affinity of clinically known small molecule bone targeting agents are analyzed and quantified for their ability to degrade.

[0176] Dialysis cells with 1ml-capacity cavities (Bel-Art Products, Pequannock, NJ) are fitted with Spectra/Por®Biotech cellulose ester dialysis membranes (Spectrum, Laguna Hills, CA) and used in drug release studies. Particles (20-50 mg) are suspended in 1 ml of saline and injected into one cavity (donor side). Fresh saline is injected into the other cavity (recipient). The cells are placed in a heated, shaking water bath (37°C). At predetermined times, the recipient solution is removed and completely replaced with fresh saline. Samples are filtered through 0.45μm syringe filters and the absorption of each is measured by HPLC. A portion of the release samples is also analyzed for the presence of mPEG-SPA, bone targeting moieties and mPEG-SPA / complexes. An HPLC size exclusion technique using Waters Ultrahydrogel columns allows the identification of the separate peaks for BSA and SSA-PEG with some overlap of the peaks. An HPLC coupled with mass spectrometry and evaporative light scattering detector allows for analysis and quantitative measurement of the bone targeting agent.

EXAMPLE 26

[0177] This example illustrates methods for evaluating the stealthiness of the nanoparticles.

[0178] The bone targeting nanoparticles are evaluated in a biological test to evaluate their detrimental potential for macrophagic engulfment as a model for the process involved in spleen and liver uptake. Briefly, the bone targeting nanoparticles loaded with pifithrin are exposed to cell cultures of the J774 macrophage cell line. At various time points the cells are separated from the supernatant by simple filtration. The filtrate is then split and one portion analyzed for the total amount of pifithrin present. The other portion of the filtrate is ultracentrifuged to separate the pifithrin still present in nanoparticles and then analyzed for soluble pifithrin. In this manner, with the proper controls the amount of pifithrin associated with the J774 cells (assumed to be phagocytized), the amount of pifithrin in solution, and the amount of pifithrin still associated with the nanoparticles is calculated. This quantitation of phagocytic potential provides an understanding of the

WHAT IS CLAIMED IS:

1. A method of modifying a cellular response in a mammal comprising administering to the mammal an effective amount of biodegradable nanoparticles, each of said nanoparticles comprising an active agent, a biodegradable polymer, and a bone targeting agent.

2. The method of claim 1, wherein modifying a cellular response comprises temporarily inhibiting a p53 protein.

3. The method of claim 1, wherein modifying a cellular response comprises activating a p53 protein.

4. The method of claim 1, wherein modifying a cellular response comprises stimulating bone marrow cells.

5. The method of claim 4, wherein the active agent is a granulocyte stimulating factor.

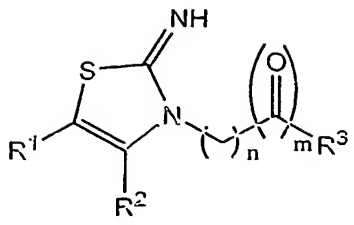
6. The method of any of claims 1-5, wherein the biodegradable nanoparticle comprises PEG and a polyester selected from the group consisting of PLA, PLGA, PGA, and mixtures thereof.

7. The method of claim 7, wherein the biodegradable nanoparticle comprises PEG and PLGA.

8. The method of claim 6, wherein the biodegradable nanoparticle comprises PLGA having a lactic acid:glycolic acid molar ratio of about 95:5 to about 5:95.

9. The method of claim 8, wherein the biodegradable nanoparticle comprises PLGA having a lactic acid:glycolic acid molar ratio of about 75:25 to about 25:75.

10. The method of claim 9, wherein the biodegradable nanoparticle comprises PLGA having a lactic acid:glycolic acid molar ratio of about 50:50.



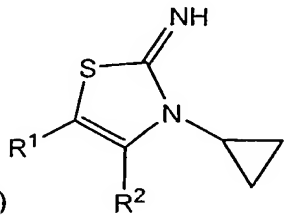
(I)

wherein m is 0 or 1, n is an integer from 1 to 4,

R^1 and R^2, taken together, form an aliphatic or aromatic carbocyclic 5- to 8-membered ring, optionally substituted with one or more straight or branched C_1-C_6 alkyl, C_1-C_6 alkoxy, hydroxy, fluoro, chloro, bromo, nitro, amino, C_1-C_6 alkylamino, and/or C_4-C_{14} aromatic or heteroaromatic moieties, and

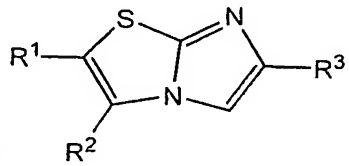
R^3 is selected from the group consisting of a C_1-C_6 alkyl group, a C_1-C_6 alkoxy group, and a phenyl group, wherein the alkyl group, the alkoxy group, or the phenyl group is optionally substituted with one or more straight or branched C_1-C_6 alkyl, C_1-C_6 alkoxy, hydroxy, fluoro, chloro, bromo, nitro, amino, C_1-C_6 alkylamino, and/or C_4-C_{14} aromatic or heteroaromatic moieties, and optionally forms a C_3-C_6 cycloalkyl when R^3 is connected to the carbon β to the thiazole ring.

23. The method of claim 22, wherein the active agent is Formula II:



(II)

24. The method of claim 2, wherein the active agent is Formula III:



(III)

wherein R^1 and R^2 taken together form an aliphatic or aromatic carbocyclic 5- to 8-membered ring, optionally substituted with one or more straight or branched C_1-C_6 alkyl,

wherein R^9, R^{10}, and R^{11} are each independently selected from the group consisting of a hydrogen, hydroxyl, methyl, fluoro, chloro, bromo, nitro, amino, methoxy, aryl, and heteroaryl.

28. The method of claim 27, wherein R^9 is methyl.

29. The method of claim 27, wherein R^9 is phenyl.

30. The method of any of claims 22 and 26-29, wherein the active agent is 2-[2-imino-4,5,6,7-tetrahydro-1,3-benzothiazol-3(2H)-yl]-1-(4-methylphenyl)-1-ethanone or 2-[2-imino-4,5,6,7-tetrahydro-1,3-benzothiazol-3(2H)-yl]-1-(biphenyl)-1-ethanone.

31. The method of claim 24, wherein the active agent is 2-p-tolyl-5,6,7,8-tetrahydro-benzo[d]imidazo[2,1-b]thiazole

32. The method of any of claims 22-31, wherein the active agent protects bone marrow cells during chemotherapy and/or radiation therapy.

33. A composition comprising an active agent, a biodegradable nanoparticle, and a bone targeting agent.

34. The composition of claim 33, wherein the biodegradable nanoparticle comprises PEG and a polyester selected from the group consisting of PGA, PLA, PLGA, and mixtures thereof.

35. The composition of claim 34, wherein the biodegradable nanoparticle comprises PEG and PLGA.

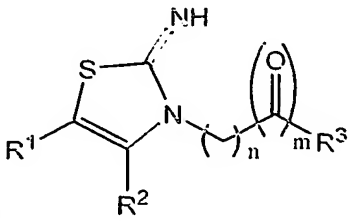
36. The composition of claim 34, wherein the biodegradable nanoparticle comprises PLGA having a lactic acid:glycolic acid molar ratio of about 95:5 to about 5:95.

37. The composition of claim 36, wherein the biodegradable nanoparticle comprises PLGA having a lactic acid:glycolic acid molar ratio of about 75:25 to about 25:75.

38. The composition of claim 37, wherein the biodegradable nanoparticle comprises PLGA having a lactic acid:glycolic acid molar ratio of about 50:50.

50. The composition of claim 49, wherein the active agent is a granulocyte stimulating factor.

51. The composition of claim 47, wherein the active agent is Formula I:



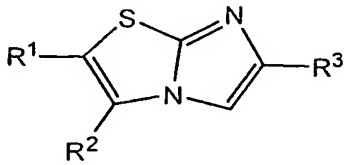
(I)

wherein m is 0 or 1, n is an integer from 1 to 4,

R^1 and R^2 taken together form an aliphatic or aromatic carbocyclic 5- to 8-membered ring, optionally substituted with one or more straight or branched C_1-C_6 alkyl, C_1-C_6 alkoxy, hydroxy, fluoro, chloro, bromo, nitro, amino, C_1-C_6 alkylamino, and/or C_4-C_{14} aromatic or heteroaromatic moieties, and

R^3 is selected from the group consisting of a C_1-C_6 alkyl group, a C_1-C_6 alkoxy group, and a phenyl group, wherein the alkyl group, the alkoxy group, or the phenyl group is optionally substituted with one or more straight or branched C_1-C_6 alkyl, C_1-C_6 alkoxy, hydroxy, fluoro, chloro, bromo, nitro, amino, C_1-C_6 alkylamino, and/or C_4-C_{14} aromatic or heteroaromatic moieties, and optionally forms a C_3-C_6 cycloalkyl when R^3 is connected to the carbon β to the thiazole ring.

52. The composition of claim 47, wherein the active agent is Formula III:



(III)

wherein R^1 and R^2 taken together form an aliphatic or aromatic carbocyclic 5- to 8-membered ring, optionally substituted with one or more straight or branched C_1-C_6 alkyl, C_1-C_6 alkoxy, hydroxy, fluoro, chloro, bromo, nitro, amino, C_1-C_6 alkylamino, and/or C_4-C_{14} aromatic or heteroaromatic moieties, and

R^3 is selected from the group consisting of a C_1-C_6 alkyl group, a C_1-C_6 alkoxy group, and a phenyl group, wherein the alkyl group, the alkoxy group, or the phenyl group is optionally substituted with one or more straight or branched C_1-C_6 alkyl, C_1-C_6 alkoxy,

63. The method of claim 62, wherein the PEG has a molecular weight of about 1000 to about 10,000.

64. The method of any of claims 55-63, wherein the bone targeting agent is selected from the group consisting of a phosphate, a phosphonate, a bisphosphonate, a hydroxybisphosphonate, an aminomethylenephosphonate, an acidic peptide, and any combination thereof.

65. The method of claim 64, wherein the biodegradable nanoparticle comprises PEG and the bone targeting agent is covalently bound to at least 10% of the PEG of the biodegradable nanoparticle.

66. The method of claim 65, wherein the bone targeting agent is covalently bound to at least 25% of the PEG of the biodegradable nanoparticle.

67. The method of claim 66, wherein the bone targeting agent is covalently bound to at least 50% of the PEG of the biodegradable nanoparticle.

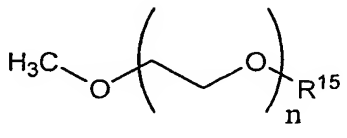
68. A process for preparing a biodegradable nanoparticle comprising an active agent, a biodegradable polymer, and a bone targeting agent, the process comprising

(1) providing an organic phase comprising one or more of (a)-(i)

(a) a biodegradable polymer,

(b) a PEG,

(c) an activated PEG of Formula VI:

(VI) 

wherein n is an integer from 2 to 2000, and R^{15} is an organic radical that contains an electrophilically activated leaving group,

(d) a bone targeting agent,

73. The process according to claim 68, wherein the organic solvent is selected from the group consisting of C_1-C_4 alcohols, C_2-C_6 esters, C_2-C_6 ethers, and C_1-C_6 organic acids.

74. The process according to claim 68 wherein the surface active agent is bovine serum albumin, human serum albumin, or polyvinyl alcohol.

75. The process according to claim 74, wherein bovine serum albumin or human serum albumin is present in the aqueous phase at a concentration of about 5-15 mg/ml.

76. The process according to claim 74, wherein the polyvinyl alcohol is present in the aqueous phase at a concentration of about 0.5 to 2.0 % by volume.

77. The process according to claim 68, wherein the organic phase comprises a biodegradable polymer and an activated PEG .

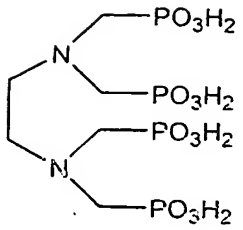
78. The process according to claim 77, wherein the organic phase further comprises a bone targeting agent.

79. The process according to any of claims 68, wherein the organic phase comprises a PEG-modified biodegradable polymer, a bone targeting agent- biodegradable polymer conjugate, and a bone targeting agent-PEG conjugate.

80. The process according to claim 68, wherein the organic phase comprises a PEG-modified biodegradable polymer, a bone targeting agent-PEG conjugate, and a bone targeting agent PEG-modified biodegradable polymer conjugate.

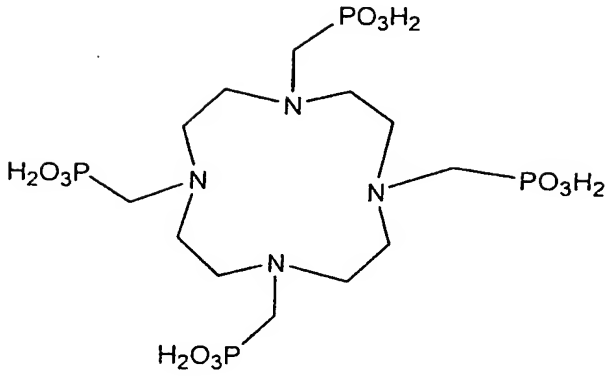
81. The process according to claim 68, wherein if an activated PEG of Formula VI is present, then R^{15} is succinimidyl propionate or succinimidyl butanoate.

82. The process according to any of claims 68-81, wherein the bone targeting agent is selected from the group consisting of a phosphate, a phosphonate, a bisphosphonate, a hydroxybisphosphonate, an aminomethylenephosphonate, an acidic peptide, and any combination thereof.



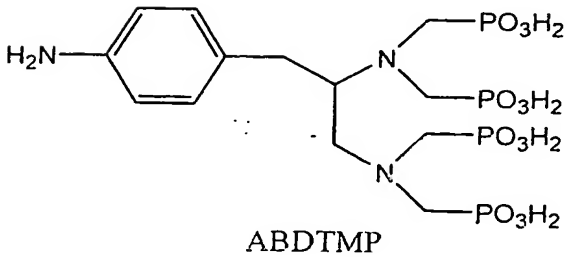
EDTMP

Figure 1



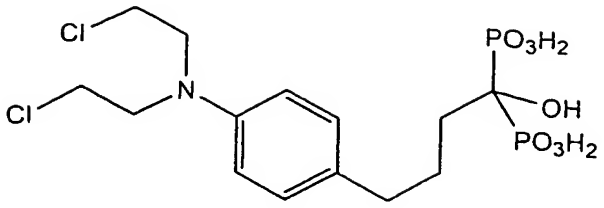
DOTMP

Figure 2



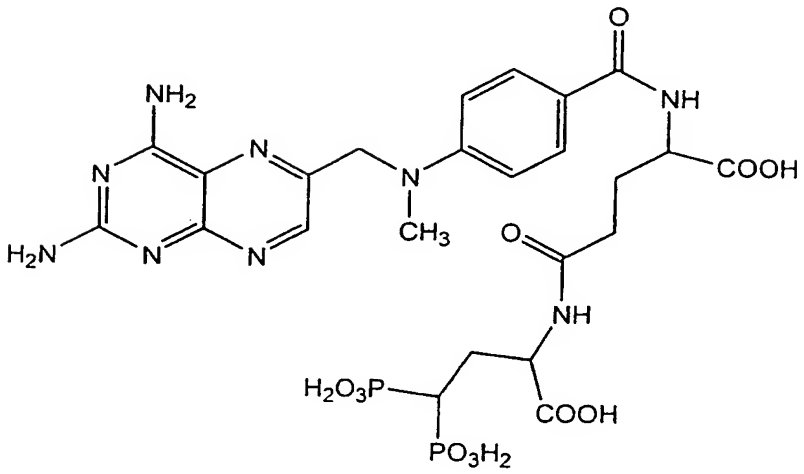
ABDTMP

Figure 3



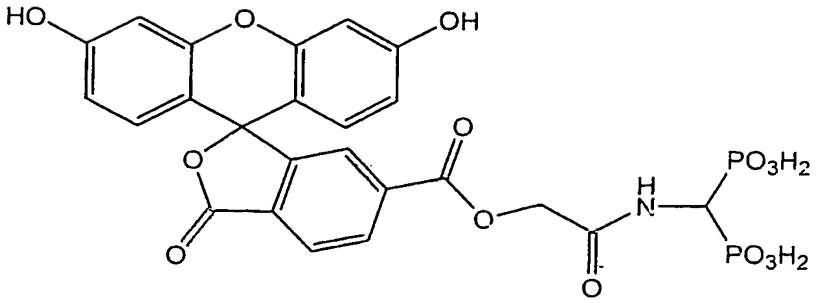
BAD

Figure 4



MTX-BP

Figure 5



CF-BP

Figure 6

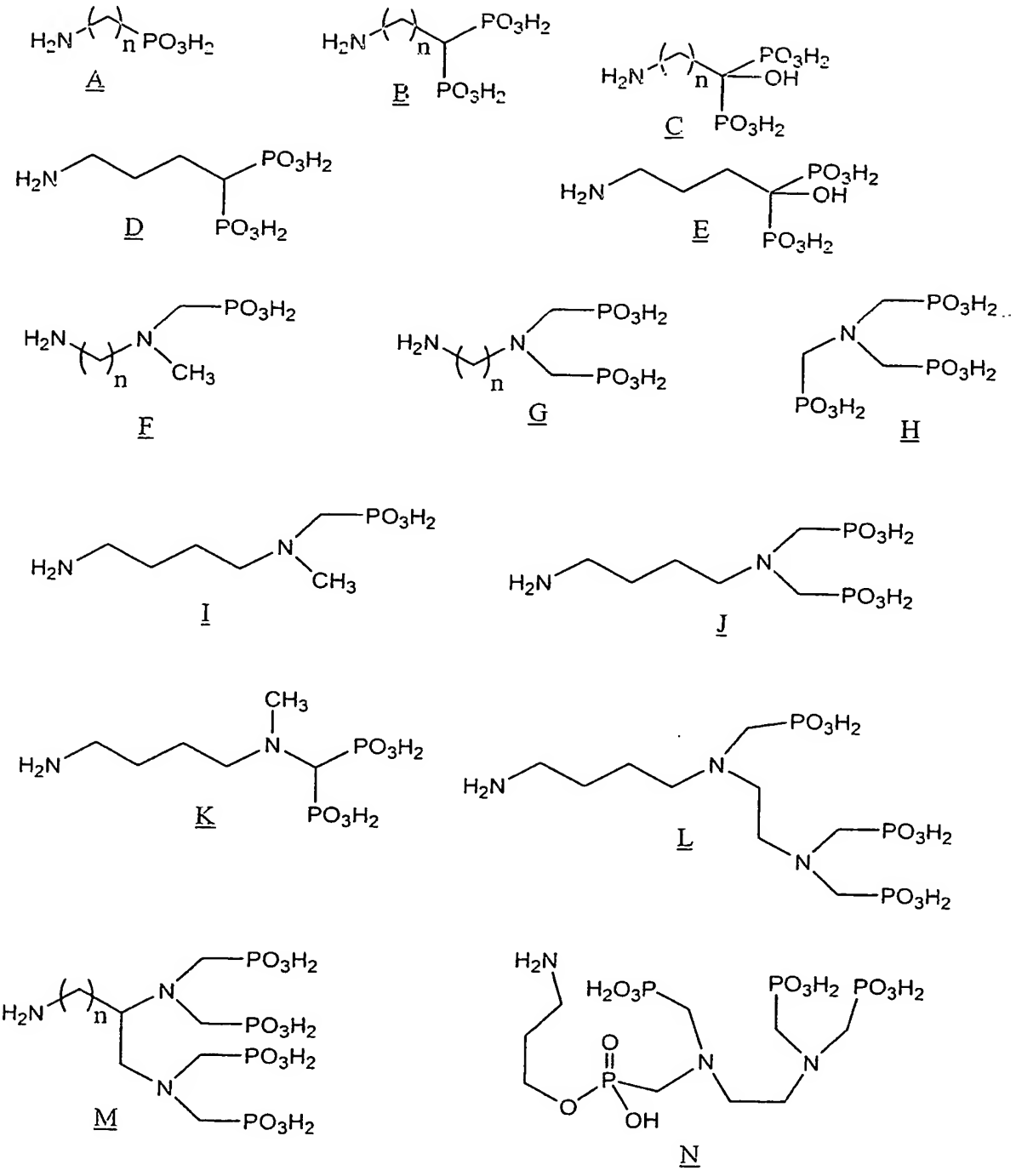


Figure 7

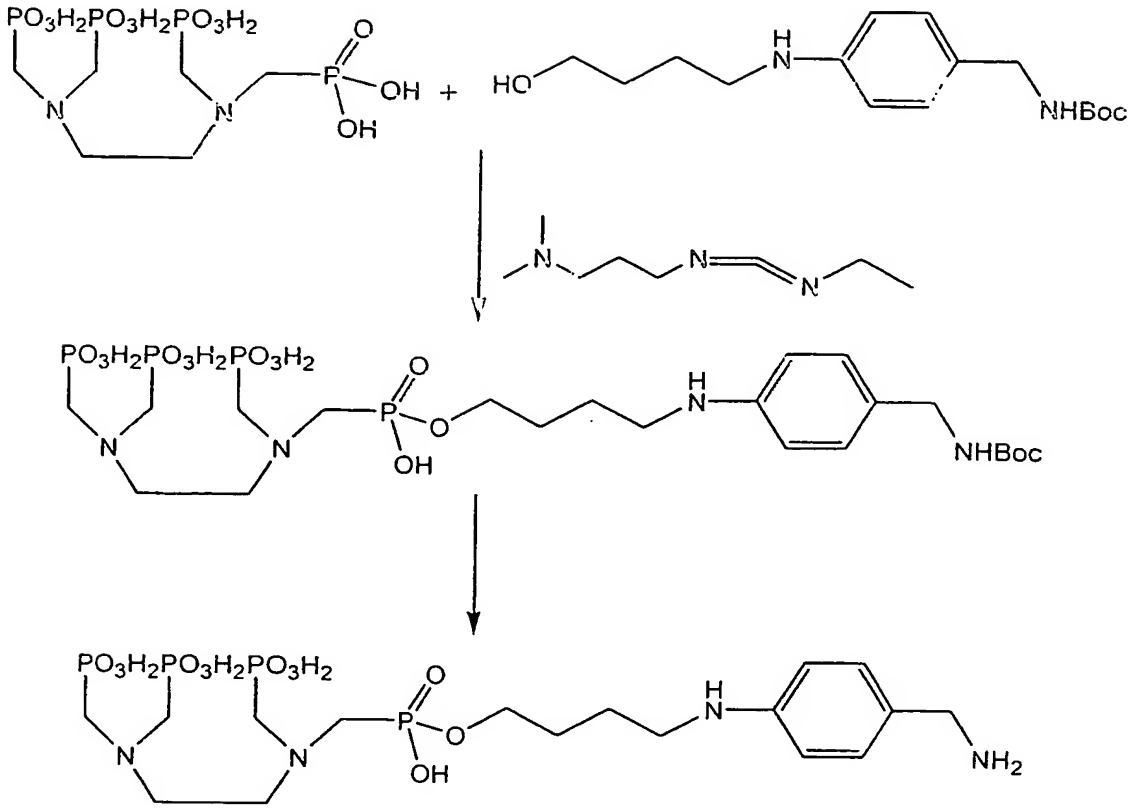


Figure 8

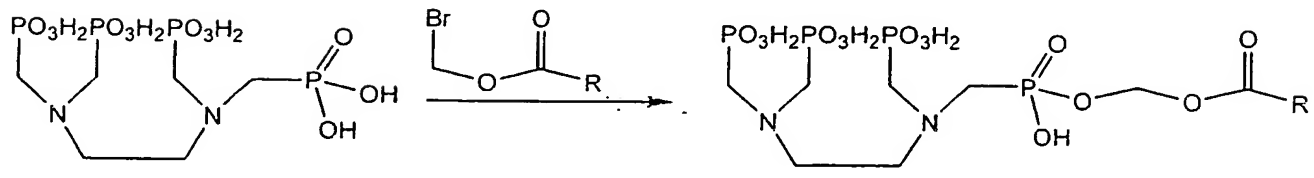


Figure 9

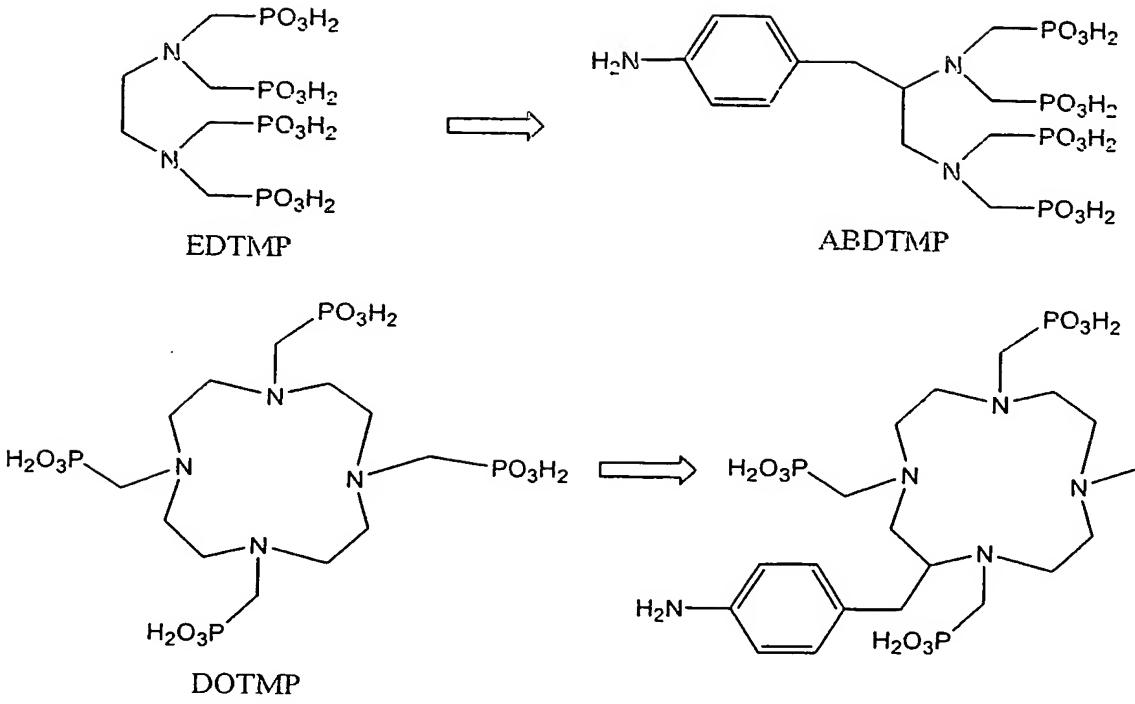
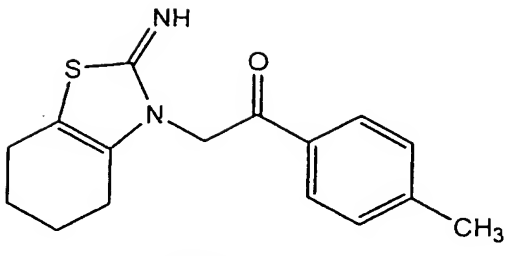
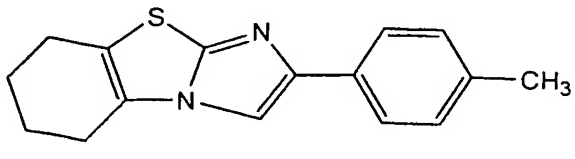


Figure 10



Pifithrin α

Figure 11



Pifithrin-β

Figure 12

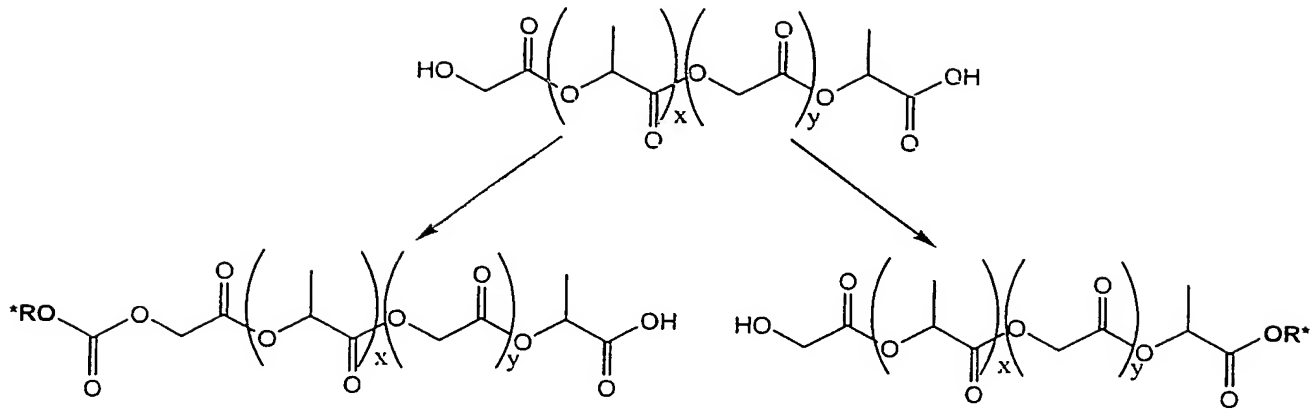


Figure 13

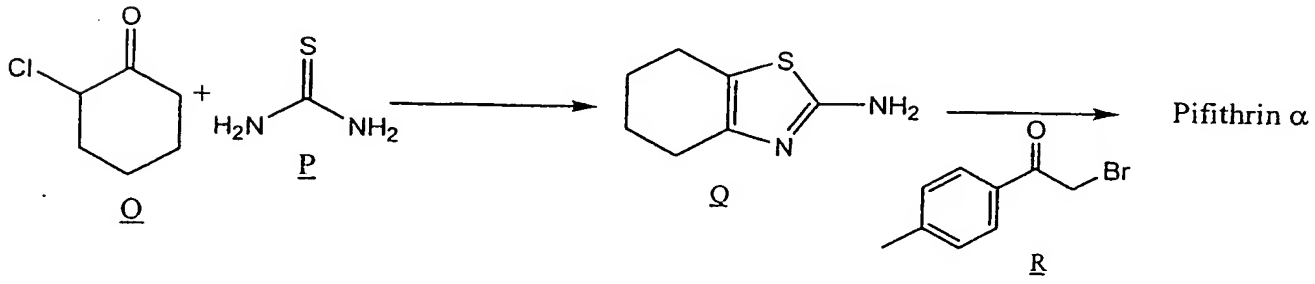


Figure 14

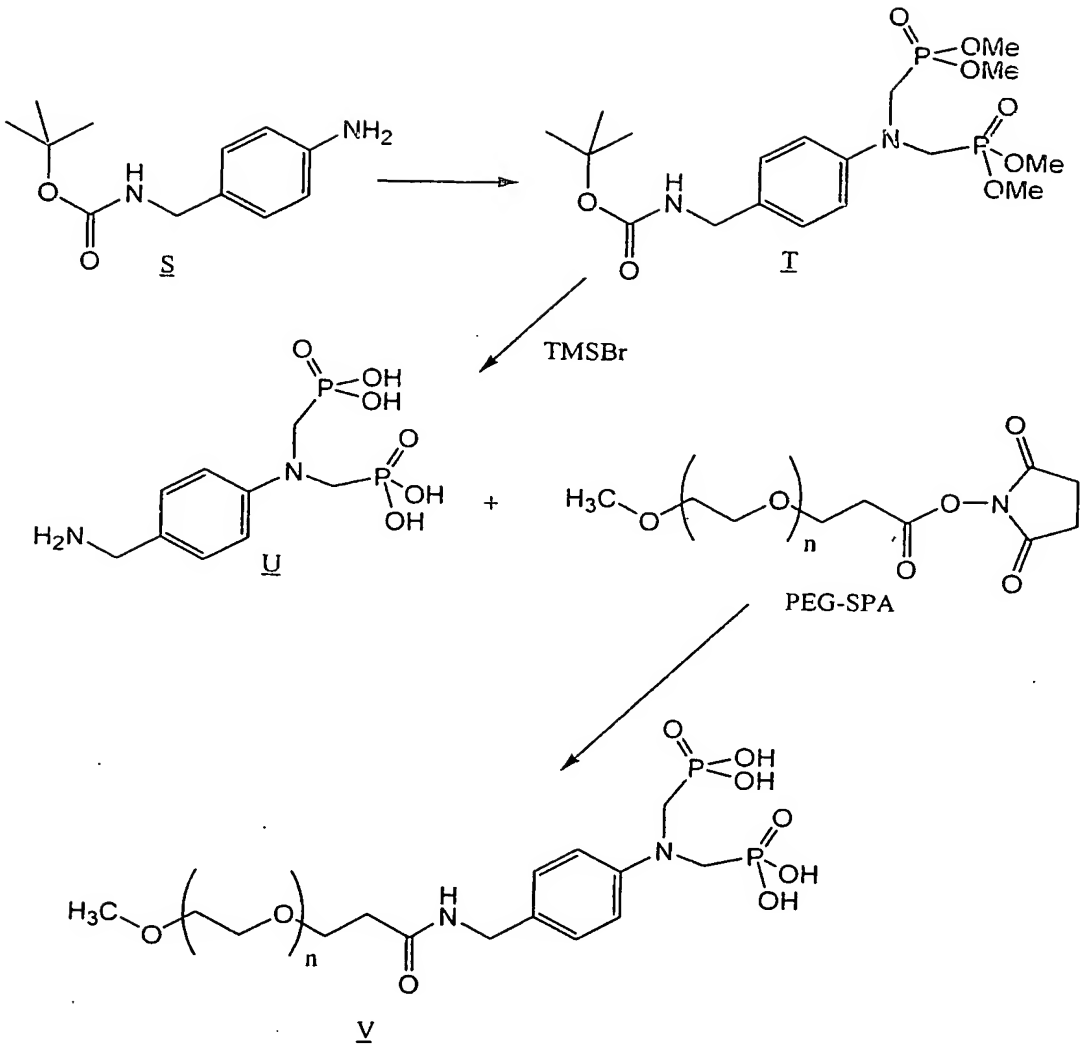
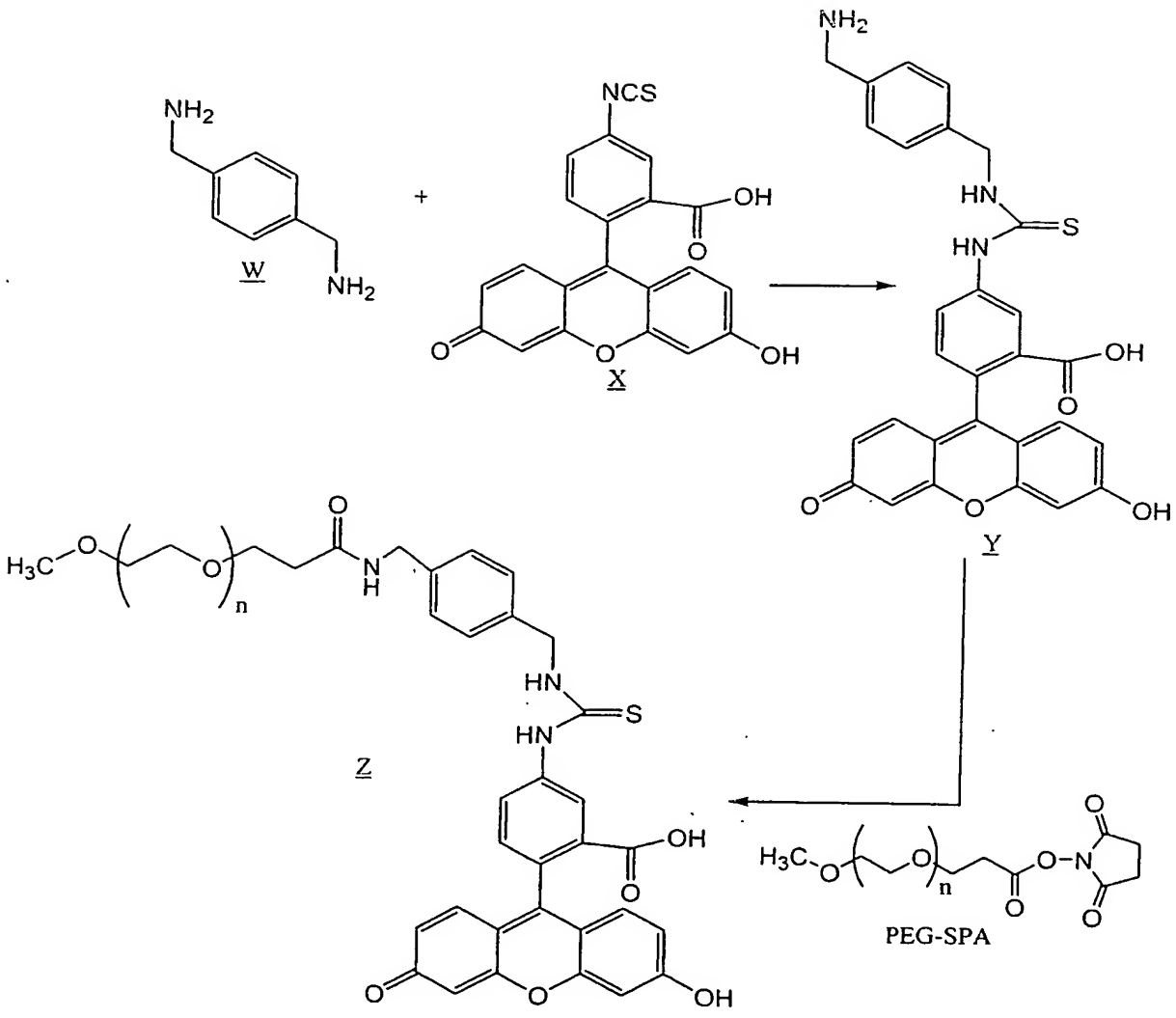


Figure 15


NH2
NH2
W
+
NCS
OH
O
O
OH
X
NH2
HN
S
HN
OH
O
O
OH
Y
H3C
O
O
n
N
H
HN
S
HN
Z
OH
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O
OH
H3C
O
O
n
O
O
N
O
O
PEG-SPA

Figure 16

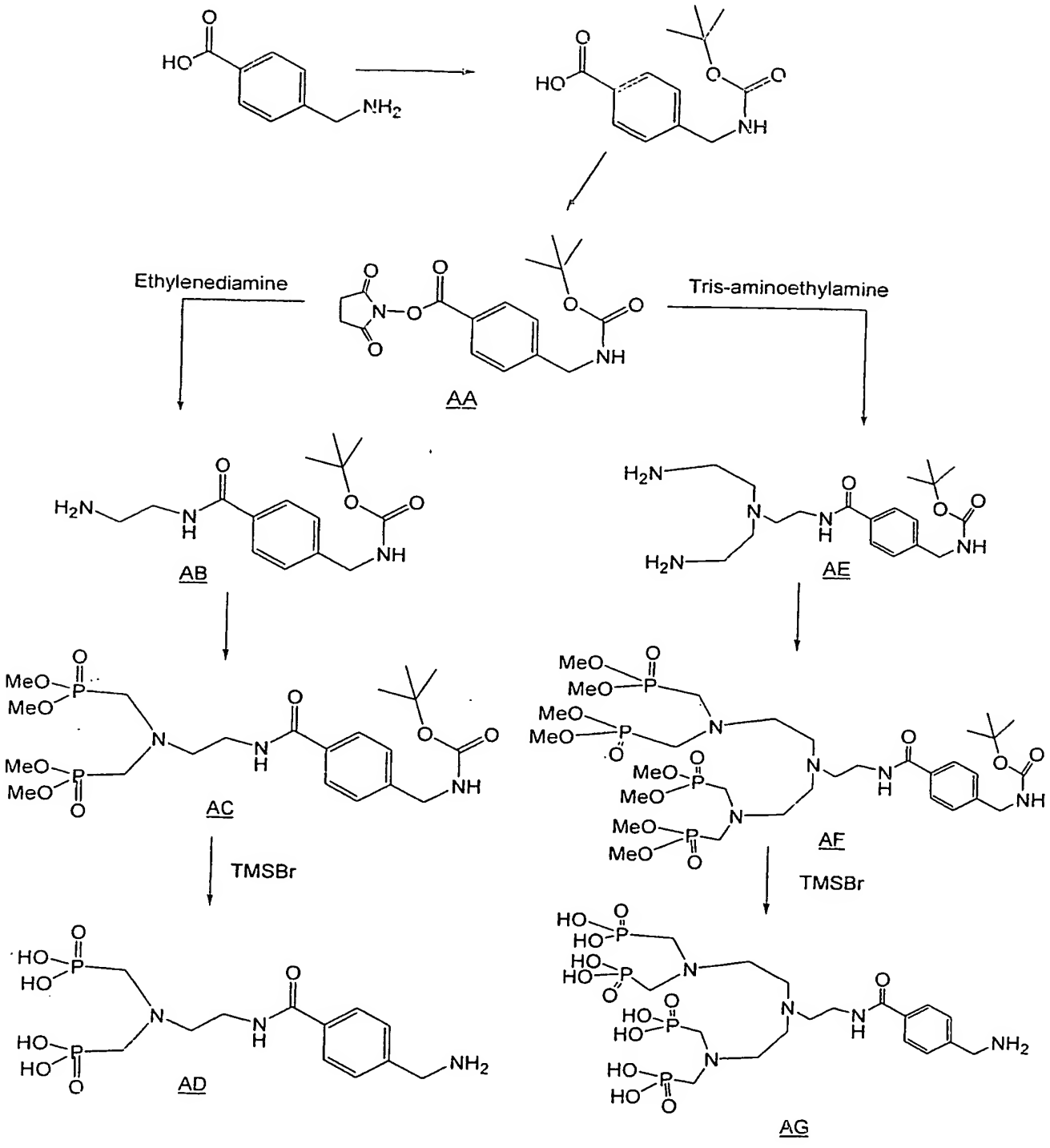
Ethylenediamine
Tris-aminoethylamine
AA
AB
AE
AC
AF
TMSBr
TMSBr
AD
AG

Figure 17

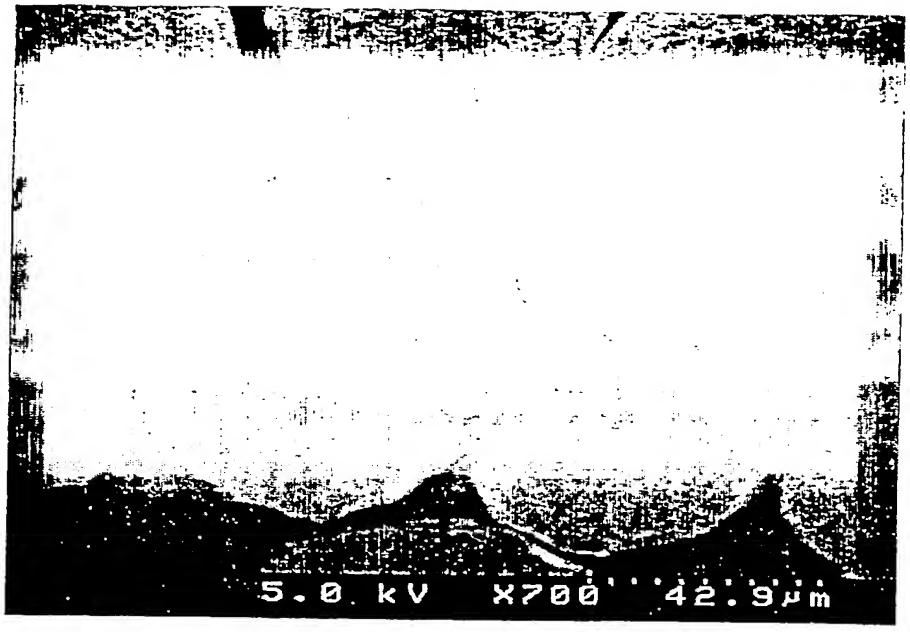

Figure 18A

Figure 18B

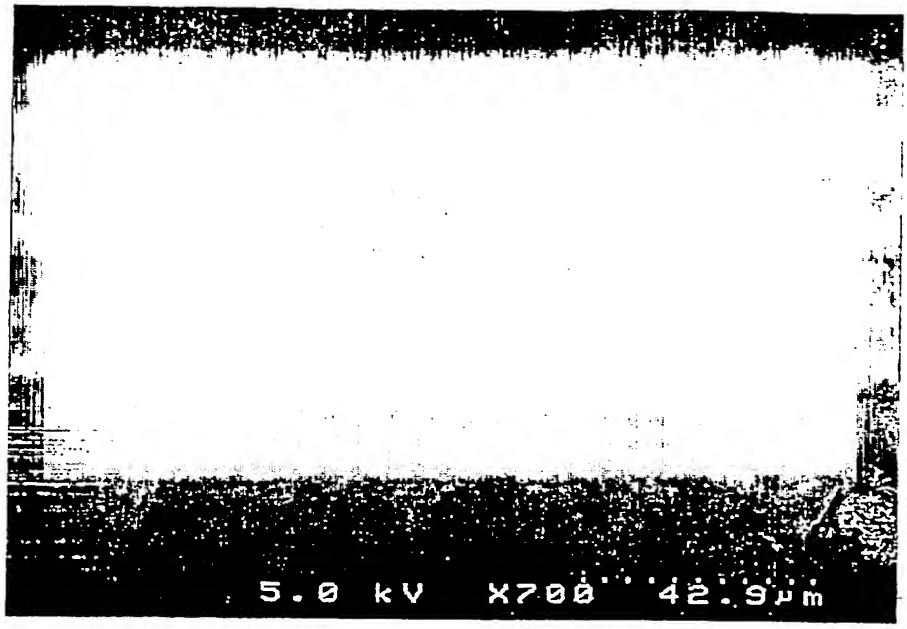

Figure 19A

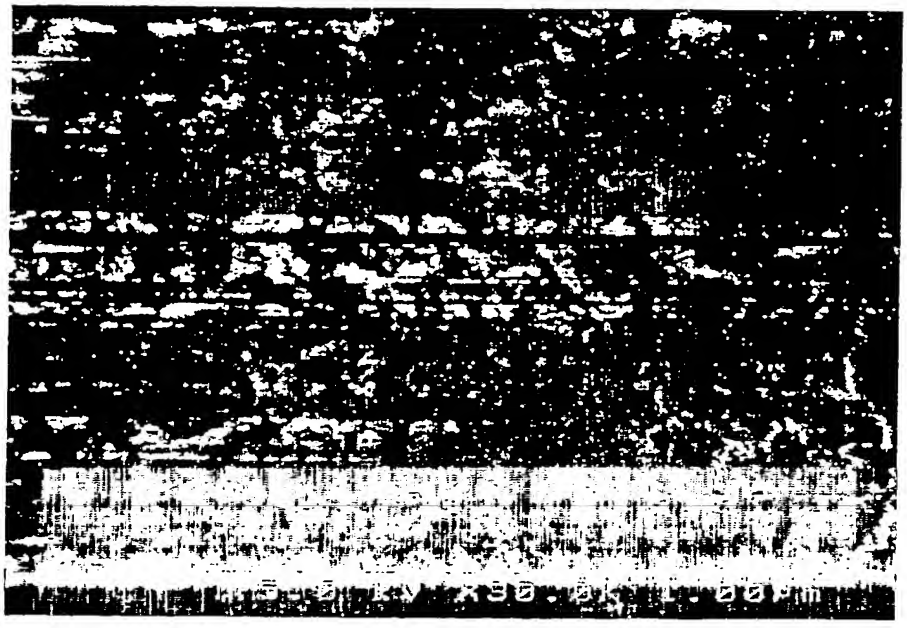

Figure 19B

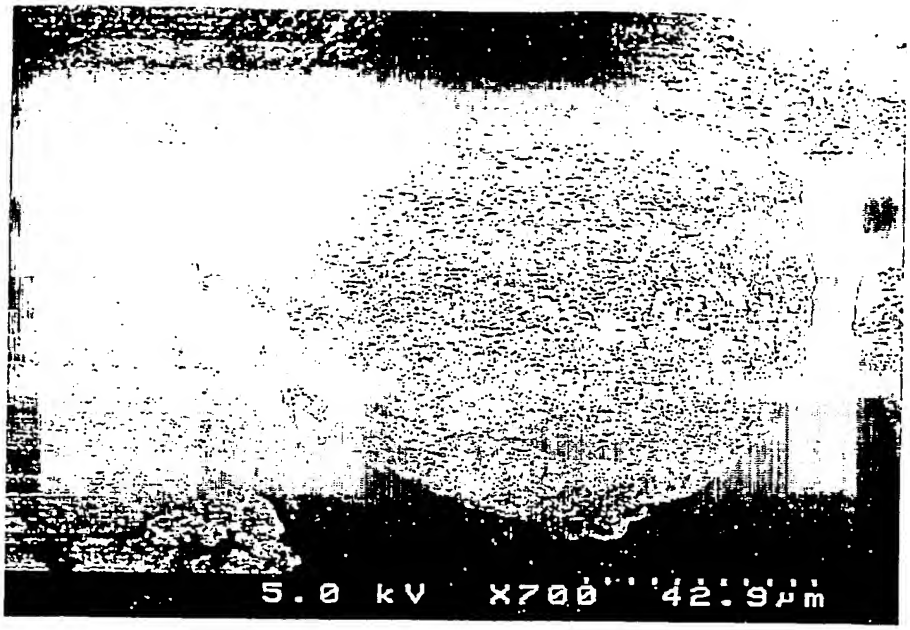

Figure 20A

Figure 20B

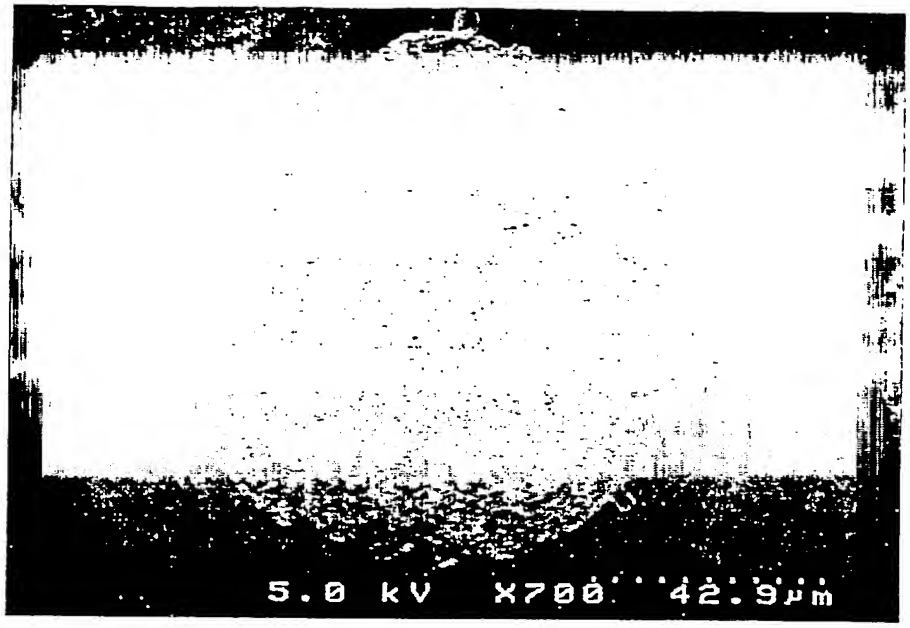

Figure 21A

Figure 21B

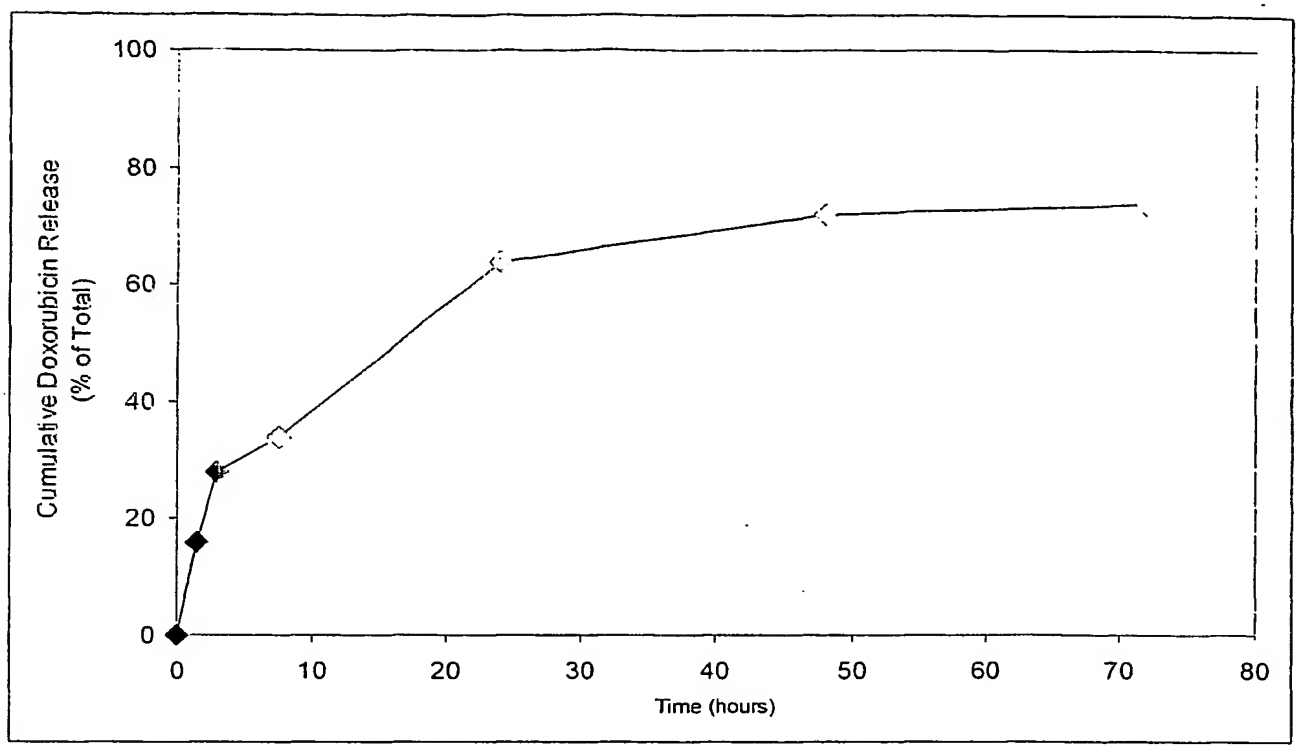

Figure 22A

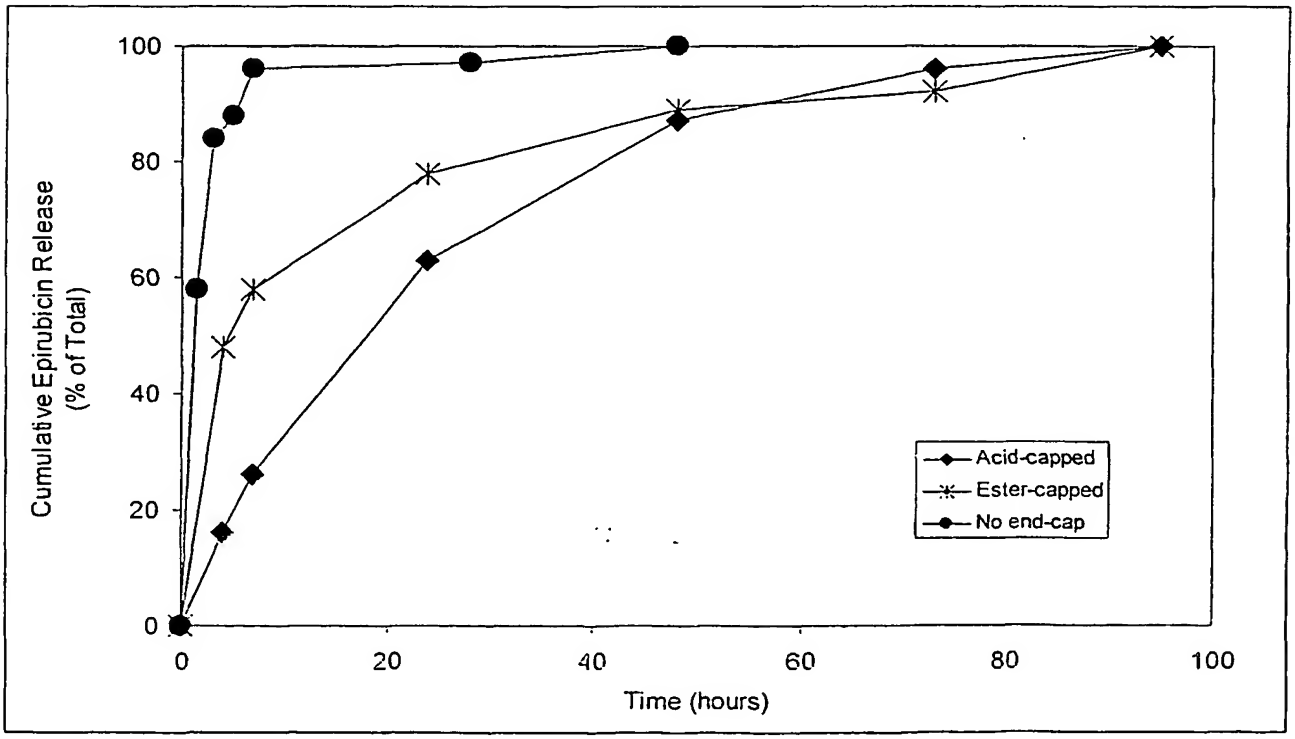

Figure 22B

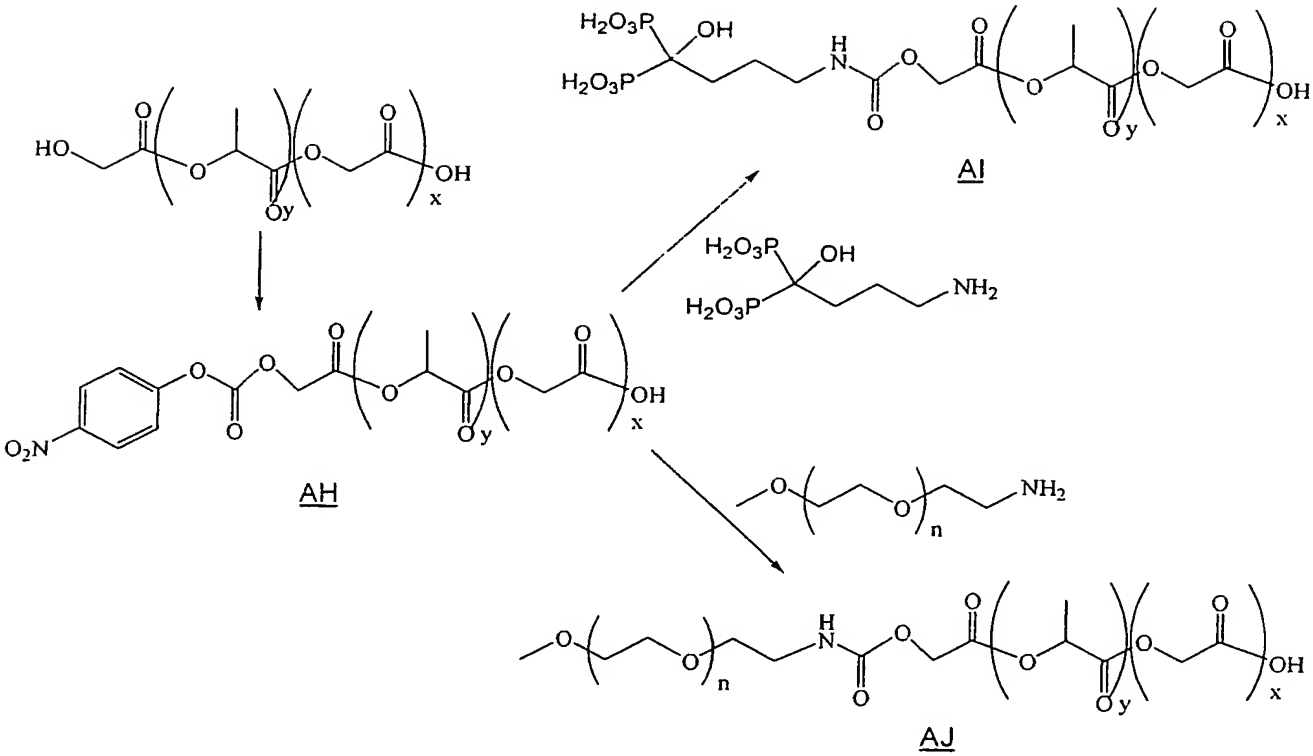

Figure 23

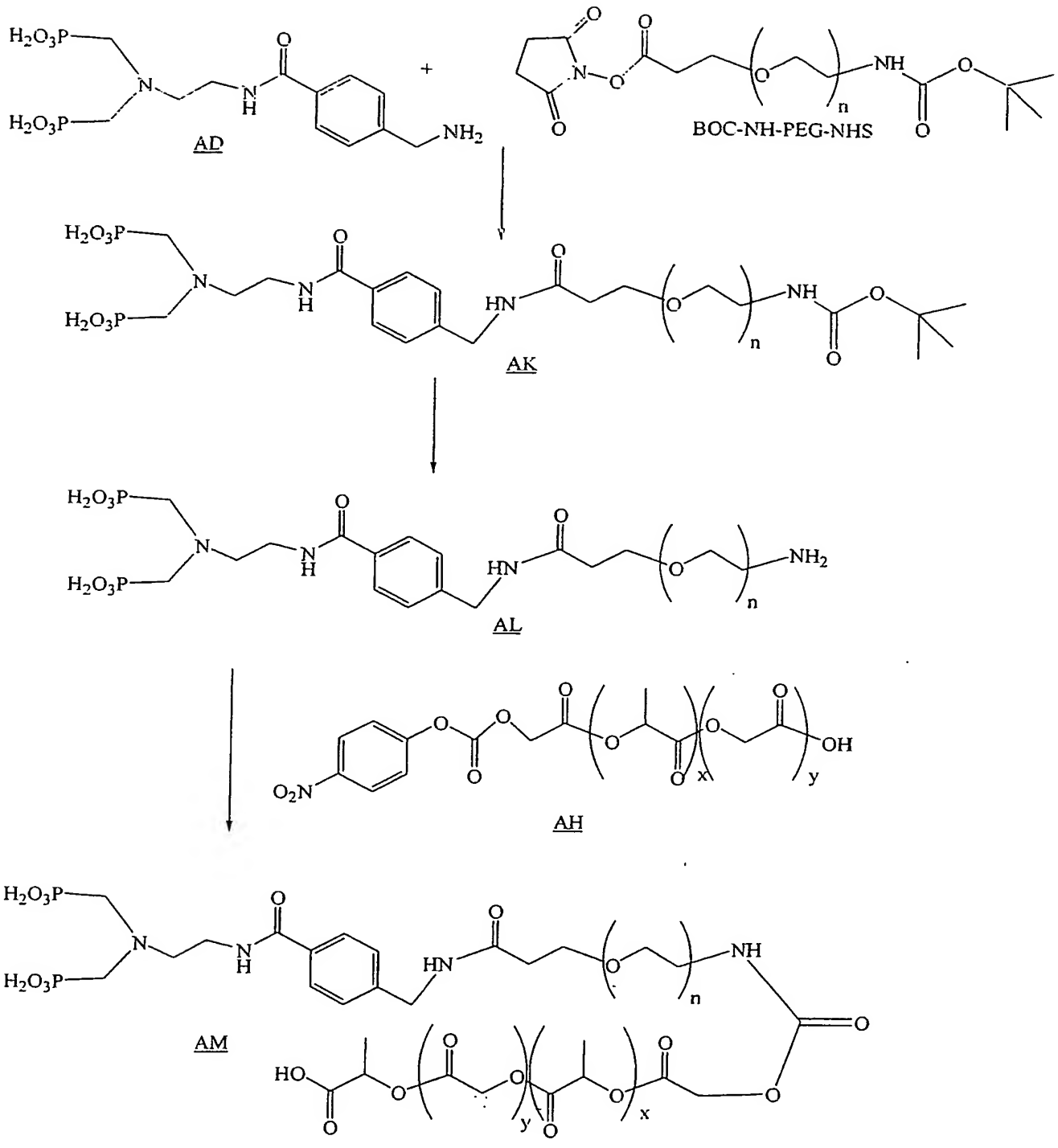

Figure 24

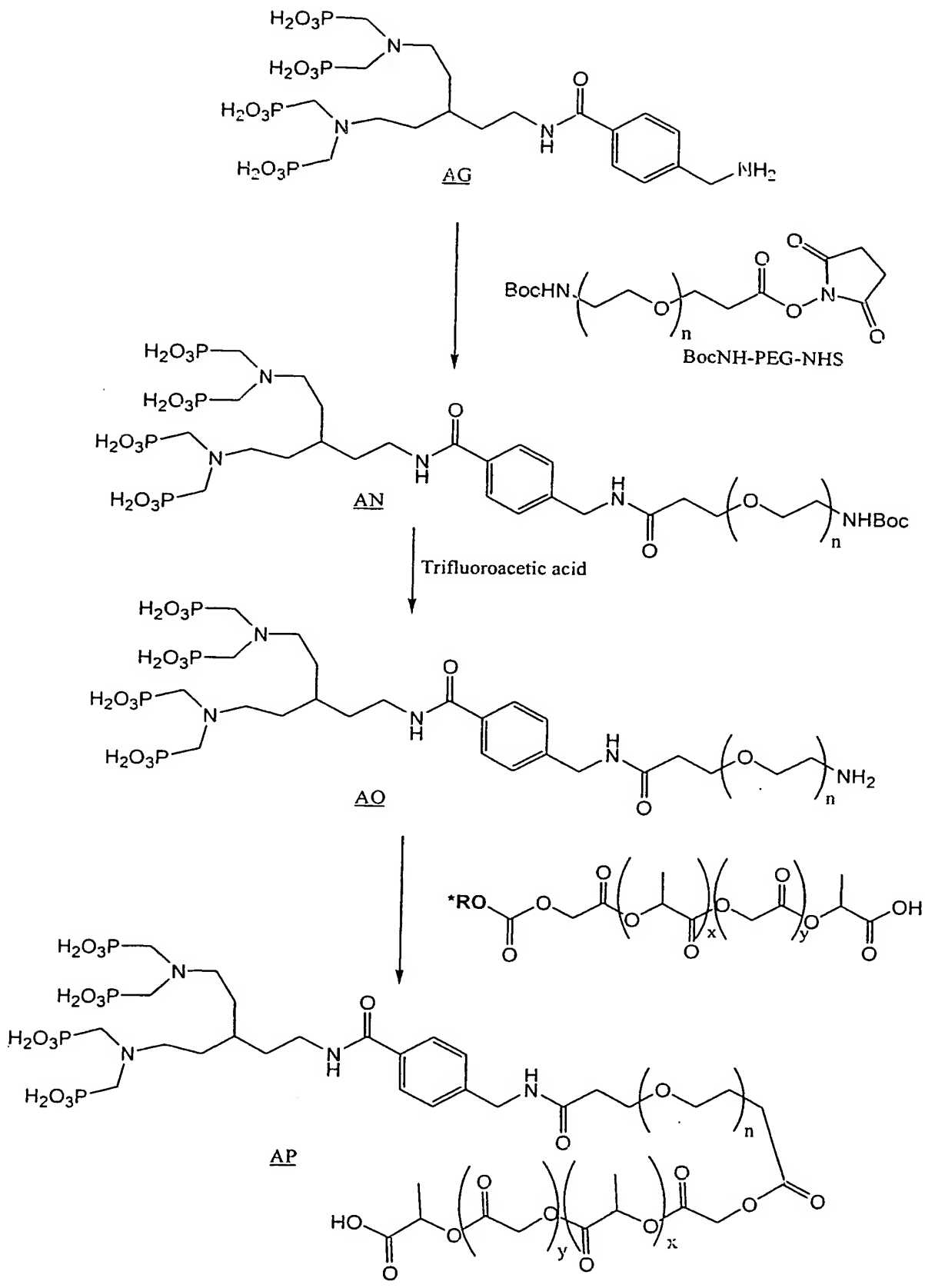

Figure 25

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US2004/010285

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K9/51 A61K47/48 A61K31/428 A61K31/427 A61K31/70

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, BIOSIS, EMBASE, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 02/083096 A (ALKERMES INC) 24 October 2002 (2002-10-24) claims 1-26 examples 1-3 page 5, line 1 - page 13, line 14	1,5-21, 33-50, 55-72, 82-87
Y	page 16, line 5 - page 20, line 25	1-87
Y	EP 0 512 844 A (CELTRIX PHARMA) 11 November 1992 (1992-11-11) claims 1-25 examples 2,3 column 4, line 50 - column 7, line 18 column 8, line 23 - column 9, line 33	1-87

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

° Special categories of cited documents :

°A° document defining the general state of the art which is not considered to be of particular relevance

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°O° document referring to an oral disclosure, use, exhibition or other means

°P° document published prior to the international filing date but later than the priority date claimed

°T° later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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°&° document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
6 September 2004	17/09/2004

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Schifferer, H

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
[redacted]T/US2004/010285

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